Vol. 35, No. 4 April, 1941

AUG 24 1941 Medical Library

THE BIOCHEMICAL JOURNAL

EDITED FOR THE BIOCHEMICAL SOCIETY

BY

C. R. HARINGTON

ASSOCIATE EDITORS

S. J. COWELL

F. DICKENS

and

F. J. W. ROUGHTON

LONDON

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, N.W. 1

CHICAGO: The University of Chicago Press

(Agents for the United States)

BOMBAY, CALCUTTA, MADRAS: Macmillan

TOKYO: Maruzen Company, Ltd.

All rights reserved

1941

Price Seven Shillings and Sixpence net

PRINTED IN GREAT BRITAIN

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted solely for the advancement of the Science of Biochemistry. Persons interested in Biochemistry are eligible for election.

Meetings are held at different centres for the communication of papers to the Society.

Members are entitled to receive a copy of the Journal. Further information as to the conditions of membership may be obtained on application to the Hon. Secs., Dr F. G. Young, National Institute for Medical Research, Hampstead, N.W. 3 or Dr W. T. J. Morgan, Lister Institute, Chelsea Bridge Road, S.W. 1, or to the Hon. Treas., Mr J. Addyman Gardner, 35 Bullingham Mansions, Church Street, London, W. 8, to whom subscriptions should be sent.

Papers for publication should be sent to Prof. C. R. Harington, F.R.S., University College Hospital Medical School, University Street, London, W.C. 1. Communications respecting the printing of the articles, or respecting the purchase of offprints should be addressed to the University Press, Cambridge.

The Journal is issued in monthly numbers and the date at which each paper is received is printed at the beginning of the paper.

All communications respecting the purchase of copies of the parts or volumes, whether current or back issues, or respecting subscriptions in the case of non-members of the Biochemical Society (£3. 10*s.* 0*d.* net per volume (post free) payable in advance) should be addressed to The Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1, or to the Hon. Treas., Mr J. Addyman Gardner, 35 Bullingham Mansions, Church Street, London, W. 8. For prices of back numbers and volumes see list on p. 4 of Wrapper.

Quotations can be given for Buckram binding cases and for binding Subscribers' Sets.

The Cambridge University Press has appointed the University of Chicago Press agent for the sale of the *Biochemical Journal* in the United States of America.

Notice to Contributors

1. Contributors who are members of the Biochemical Society receive 25 copies (without covers) of their papers free and can purchase additional copies if notice be given when their proofs are returned. Other contributors may purchase reprints but receive no free copies.

2. Papers submitted for publication are understood not to be offered to any other Journal for prior or simultaneous publication.

3. All communications intended for publication must be type-written.

4. Contributors residing outside Europe are requested to nominate someone resident in Great Britain to be responsible for the correction of the proofs of their papers. Papers from such contributors should be accompanied by a statement of the number of reprints required.

5. **References.** References to literature should be arranged alphabetically according to authors' names at the end of the communication, each accompanied by the date, title of Journal, volume and page, thus: Ackermann (1912). *Z. Biol.* **59**, 17. Titles of Journals should be abbreviated in accordance with the "World List of Scientific Periodicals". In the text the reference should be indicated by the author's name and the date, enclosed in brackets, thus [Ackermann, 1912]. If reference is made to several papers published by one author in a single year, these should be numbered in sequence and the number quoted along with the year both in the text and in the collected references.

6. Chemical formulae should be written, as far as possible, in a single horizontal line.

7. Illustrations and curves accompanying the papers must be carefully drawn, about twice the size of the finished block, on smooth white Bristol boards or ordinary curve paper in Indian ink. Any lettering on these drawings should be lightly inserted in pencil. Legends should be typed on slips of paper attached to the drawings.

During 1941 the Journal will be issued at as frequent intervals as possible. MSS. for the successive numbers of Vol. 35 should be received by the first of each month. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

44. DIFFUSING FACTORS

7. CONCENTRATION OF THE MUCINASE FROM TESTICULAR EXTRACTS AND FROM *CROTALUS ATROX* VENOM

BY J. MADINAVEITIA[1]

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

FRACTIONATION of testicular extracts by lead acetate at different *p*H [Morgan & McClean, 1932; Madinaveitia, 1938] and by $(NH_4)_2SO_4$ at various concentrations [Madinaveitia, 1939, 1] are convenient methods for the concentration of diffusing factors from these sources. There is not complete parallelism between the mucolytic and diffusing activities of concentrates obtained by either of these two methods [Madinaveitia *et al.* 1940]. However, by a combination of both procedures highly active preparations of mucinase have now been obtained. The relatively accurate viscosimetric assay method [Madinaveitia & Quibell, 1940] for mucolytic activity has made it possible to determine with some exactitude the optimal *p*H range for lead acetate purification. The best mucinase preparations obtained by this method were about 1000 times more active than the dry testicle powder used as a starting material.

From a dry testicle powder *M* NaCl in *M*/10 acetic acid extracts about the same amount of mucinase as *M*/10 acetic acid alone. The precipitate formed on dilution of the NaCl extract with water contains the whole of the mucinase and the diffusing factor present in the original extract. This is probably due to adsorption of the active constituents on the protein precipitate which occurs as the electrolyte concentration is decreased. The mucinase from preparations purified with $(NH_4)_2SO_4$ does not precipitate in this way and the purest preparations of mucinase and of diffusing factor are soluble in distilled water. In this precipitation we again observe the mucinase and the diffusing factor going together in a fractionation.

The substrate used throughout this paper for testing the mucinase activity [Madinaveitia & Quibell, 1940] was a preparation of vitreous humour. Following the original idea of Chain & Duthie [1940] that diffusing factors are mucinases, Mayer *et al.* [1940, 1, 2] have suggested that the substrate on which these factors act in the skin is similar to, if not identical with, the polysaccharide from the cornea. However, intracorneal injections of diffusing factor preparations into the isolated eyes of slaughtered cattle do not increase the area of spread relative to control injections.

There is evidence that the diffusing factor and the neurotoxic constituents of *C. terrificus* are different [Madinaveitia, 1939, 2]. The mucolytic action of *C. atrox* venom has now proved to be due to a more thermolabile constituent than either the protease or the lecithinase of this venom. Fuller's earth readily adsorbs the mucinase from dialysed testicular extracts, but a relatively large amount of adsorbent is required for removal of the enzyme from solutions of *C. atrox* venom. In this case it is possible to remove part of the inactive material by adsorption at suitable *p*H with a small amount of fuller's earth and to produce

[1] Beit Memorial Research Fellow.

a further inactive precipitate by readjustment of the *p*H. $(NH_4)_2SO_4$ fractionation can also be used for purification of the mucinase from the snake venom, the active constituents precipitating at about half-saturation.

Experimental

Purification of testicular mucinase

$(NH_4)_2SO_4$ fractionation. Dry testicle powder [Madinaveitia, 1938] (500 g.) mixed with sand (1 kg.) was extracted in a mortar with acetic acid (5 lots of 500 ml. *M*/10). The extract was filtered through paper and the residue twice extracted with acetic acid (2 lots of 1 l. *M*/10). To the pooled filtered extracts (4 l.; *p*H 3·7) $(NH_4)_2SO_4$ was added to 27 % of saturation. The inactive precipitate was removed and the filtrate made 70 % saturated with $(NH_4)SO_4$. The precipitate now formed contained practically the whole of the mucinase. It was dissolved in water (200 ml.) and dialysed in a cellophane bag for 24 hr. against running tap water. The dialysed mucinase solution (1 l.) was concentrated *in vacuo* (37°) and the concentrated solution (100 ml.) evaporated to dryness whilst frozen. Yield: 22 g. having 85 % of the mucinase of the original material.

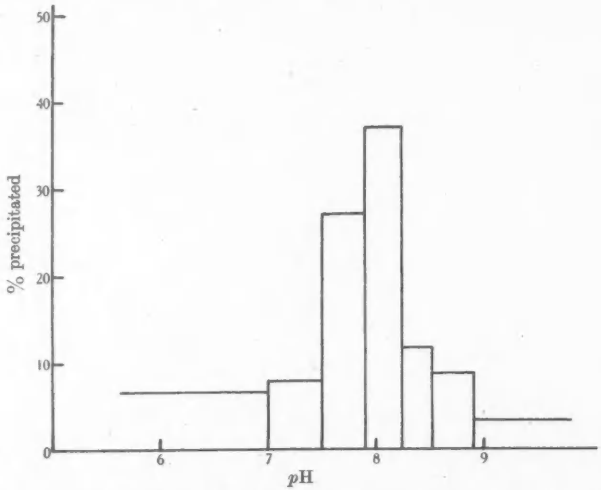


Fig. 1. Lead acetate precipitation of testicular mucinase at different *p*H.

Lead acetate fractionation. To a solution (*p*H 4·37) of $(NH_4)_2SO_4$-purified mucinase (2 g.) in water (5 ml.) a solution of neutral lead acetate (2 ml. 10 %) was added. To this opalescent mixture (*p*H 5·2) dilute ammonia (*M*/5) was added in small portions; the *p*H was determined electrometrically and the precipitate removed before addition of further amounts of ammonia. Each of the precipitates was dissolved in acetic acid (2 ml. *M*/10) and all of them made up to the same volume (50 ml.) with water. Fig. 1 represents the relative mucolytic activities of these precipitates.

A larger amount (10 g.) $(NH_4)_2SO_4$-purified mucinase was dissolved in water (250 ml.) and to the solution neutral lead acetate (100 ml. of 10 %) was added.

The *p*H was then adjusted to 7·37 with ammonia (2*N*) and the precipitate discarded. The filtrate was adjusted to *p*H 8·55 with further amounts of ammonia. The precipitate now formed was collected, dissolved in acetic acid (50 ml. *M*/10) and dialysed for 18 hr. in a cellophane bag against running tap water. After filtering from an inactive precipitate the dialysed solution was evaporated to dryness whilst frozen. The resulting preparation (0·7 g.) had 80 % of the mucinase of the already purified starting material. Lead acetate in the concentrations used in the test has no effect on the action of the mucinase on the substrate.

NaCl fractionation. Dry testicle powder (1 g.) mixed with sand (2 g.) was extracted in a mortar with a mixture of NaCl (18 ml. *M*) and acetic acid (2 ml. *M*). After 15 min. the insoluble constituents were centrifuged off. The slightly turbid supernatant (1 ml.) was diluted with water (19 ml.) and kept for 1 hr. Precipitate and supernatant were now separated and the former dissolved in NaCl (20 ml. *M*). Dilutions (1/20) of each of the fractions were tested for mucolytic and diffusing activities [Madinaveitia, 1938, 1939, 1].

	Mucolytic activity. % of original solution	Diffusing activity. Area of spread in cm.²	Increase of the area of spread
Original extract	100	8·31	4·29
Supernatant	5	4·81	0·79
Precipitate	75	7·58	3·56
Control	—	4·02	—

To a solution of $(NH_4)_2SO_4$-purified mucinase (300 mg.) in water (12 ml.) solid NaCl (2 g.) was added. On addition of acetic acid (0·5 ml. *M*) a precipitate was formed (precipitate i) which, after 10 min., was collected by centrifuging. In the supernatant a further amount of NaCl (1 g.) was dissolved and the precipitate now formed (precipitate ii) collected as before. Each of the precipitates was dissolved in water (10 ml.), their N contents established and dilutions in water of each precipitate (1/10) and the final supernatant (1/50) were tested for mucolytic activity, no precipitate appearing on dilution.

	% of original solution	
	N	Mucinase
Precipitate i	28·5	5
Precipitate ii	38·5	8
Supernatant	24·5	86

Heat-inactivation of Crotalus atrox *venom*

Crude dried *C. atrox* venom (0·5 g.) dissolved in water (25 ml.) was heated for 10 min. in thermostats at different temperatures. Each sample was then made isotonic by addition of the appropriate volume of 8·5 % NaCl. The lecithinase activity was estimated by the method of Slotta & Szyszka [1938]. Proteolytic activity was then estimated by allowing the venom solution (1 ml.) to react with gelatin (10 ml. 5 %) at 37° and determining the degree of hydrolysis by formaldehyde titration.

Temperature heated ° C.	Lecithinase activity. Dilution of enzyme					Proteolytic activity increase ml. *M*/50 KOH		Mucinase half life-time min.
	1/100	1/200	1/400	1/800	1/1600	4 hr.	18 hr.	
	Haemolysis							
Unheated	+	+	+	+	±	0·14	0·25	7
45°	+	+	+	+	−	0·15	0·25	14·5
55°	+	+	+	+	−	0·12	0·25	∞
65°	+	±	±	−	−	0·14	0·25	—

Purification of the mucinase of Crotalus atrox *venom*

Solutions of the venom (12 mg.) in water (12 ml.) are turbid. The insoluble material is brought into solution by addition of acetic acid (*M*/10), Na_2HPO_4 (*M*/15) and to a less extent by NaCl (*M*/3). The water-insoluble constituents are inactive and readily flocculated at *p*H 5·1 (*M*/15 acetate buffer).

$(NH_4)_2SO_4$ fractionation. A solution of the dry crude venom (1·65 %) was centrifuged and to the supernatant (4 ml.) acetic acid (0·2 ml. *M*) were added. To this solution (2 ml.) increasing amounts of saturated $(NH_4)_2SO_4$ solution were added, the precipitate being removed after each addition. The precipitates were dissolved in *M* NaCl (10 ml.) and tested for mucolytic activity:

% saturation in $(NH_4)_2SO_4$	0–33	33–50	50–67
Mucolytic activity % of original solution	21	62	25

Dry *C. atrox* venom (1 g.) dissolved in water (50 ml.) was acidified with acetic acid (2·5 ml. *M*); saturated $(NH_4)_2SO_4$ (25 ml.) was added and the precipitate discarded. To the clear solution more $(NH_4)_2SO_4$ (75 ml. saturated solution) was added and the active precipitate collected. This precipitate was extracted with water (20 ml.), the extract acidified with acetic acid (0·2 ml. *M*) and again precipitated with $(NH_4)_2SO_4$ (5 ml. saturated solution). The inactive precipitate was discarded and the clear solution treated with more $(NH_4)_2SO_4$ (30 ml. saturated solution). The active precipitate now formed was collected, dissolved in water (25 ml.) dialysed in a cellophane bag against running tap water overnight and dried whilst frozen. Yield: 0·07 g. of a preparation having 40 % of the mucinase content of the starting material (1 g. crude venom).

Adsorption of C. atrox *mucinase.* A solution (0·5 %) of crude venom was centrifuged. To part of the supernatant (20 ml.) acetic acid (1 ml. *M*) was added (*p*H 3·5) and another portion (20 ml.) was diluted with water (1 ml.) (*p*H 6·05). Samples (0·5 ml.) of each of these two solutions were shaken with different adsorbents (0·2 g.). After 30 min. the adsorbates were centrifuged and the N and mucinase contents of the supernatant estimated:

		% of original solution	
*p*H	Adsorbent	N	Mucinase
3·5	Kaolin	28	39
	Kieselguhr	34	18
	Fuller's earth	48	12
6·0	Kaolin	41	0
	Kieselguhr	34	10
	Fuller's earth	48	10

Adsorption with fuller's earth. A centrifuged solution (50 ml. 0·5 %) of crude *Crotalus* venom was acidified with acetic acid (1·25 ml. *M*). Samples of the solution (5 ml.) were shaken with various amounts of fuller's earth and kept for 30 min. with occasional stirring. After removal of the adsorbates the N and mucinase contents of the supernatants were estimated:

g. Fuller's earth	0·8	0·4	0·2
% N adsorbed	96	77	48
% Mucinase adsorbed	100	66	25

To samples (5 ml.) of a solution of crude venom (5 mg./ml.) acetate buffers (1 ml. *M*) of different *p*H were added and each sample adsorbed with fuller's

earth (0·25 g.). The adsorbate was removed and dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 tested for mucolytic activity. The N contents of the supernatants were also determined. The results are recorded in Fig. 2.

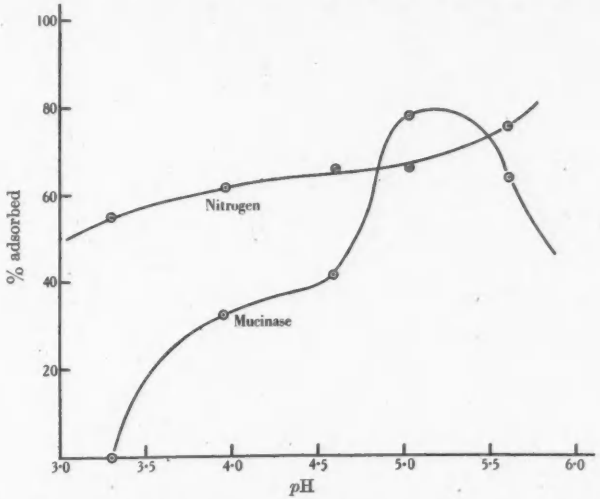


Fig. 2. Effect of *p*H on the adsorption of snake venom mucinase on fuller's earth.

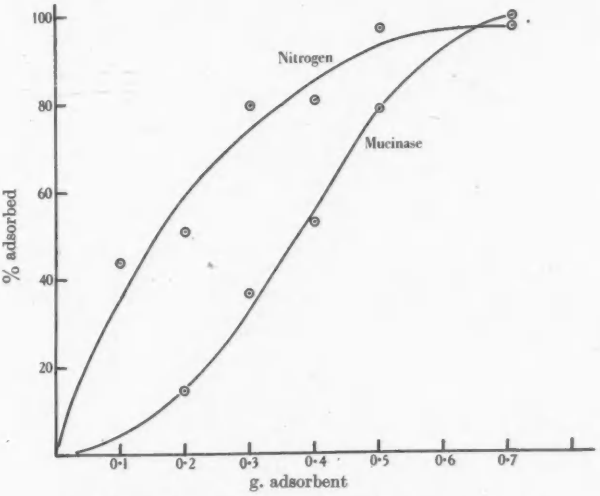


Fig. 3. Effect of the amount of adsorbent on the adsorption of snake venom mucinase on fuller's earth.

A solution (5 mg./ml.) of crude venom was mixed with acetate buffer (*M*). Samples of the buffered solution (*p*H 3·7) were adsorbed with various amounts of

fuller's earth and after 30 min. the adsorbates were removed. Dilutions (1/3) of the supernatant in *M* citrate buffer *p*H 4·7 were tested for mucolytic activity, and the N contents of the supernatants determined. The results are recorded in Fig. 3.

A solution of crude venom (60 ml., 5 mg./ml.) was mixed with acetate buffer (5 ml. *M*). The mixture (*p*H 3·5) was shaken with fuller's earth (2·5 g.) and filtered after 15 min. Samples (10 ml.) of the filtrate were partially neutralized with NaOH (1 ml.) of increasing concentration. The precipitate now formed was centrifuged after 1 hr. and the amounts of mucinase and N precipitated were determined.

	*p*H ...	4·50	5·25	6·00
% precipitated:	Mucinase	0	0	20
	N	50	50	70

Summary

$(NH_4)SO_4$-fractionation of aqueous testicular extracts, followed by lead acetate precipitation under controlled *p*H, gives a convenient purification of mucinase.

This enzyme can also be purified by dilution of NaCl extracts of testicle with water. Both mucolytic and diffusing activities are in the precipitate. The lecithinase and protease of *Crotalus atrox* venom are more thermostable than the mucinase. Concentration of the mucinase from this venom has been carried out by $(NH_4)_2SO_4$-fractionation and by adsorption methods.

The author's thanks are due to Prof. A. R. Todd for his continued interest and advice. He is also grateful to Imperial Chemical Industries, Ltd., for grants.

References

Chain & Duthie (1940). *Brit. J. exp. Path.* **21**, 324.
Madinaveitia (1938). *Biochem. J.* **32**, 1806.
—— (1939, 1). *Biochem. J.* **33**, 347.
—— (1939, 2). *Biochem. J.* **33**, 1470.
—— & Quibell (1940). *Biochem. J.* **34**, 625.
—— Todd, Bacharach & Chance (1940). *Nature, Lond.*, **146**, 197.
Mayer & Chaffee (1940, 1). *Proc. Soc. exp. Biol., N.Y.*, **43**, 487.
—— Hobby, Chaffee & Dawson (1940, 2). *Proc. Soc. exp. Biol., N.Y.*, **44**, 294.
Morgan & McClean (1932). *J. Soc. chem. Ind., Lond.*, **51**, 912.
Slotta & Szyszka (1938). *Ber. dtsch. chem. Ges.* **71**, 258.

45. STUDIES ON DIFFUSING FACTORS

8. THE REDUCTION OF THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS BY ASCORBIC ACID AND SOME DIAZO COMPOUNDS

By J. MADINAVEITIA[1] and T. H. H. QUIBELL

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

The work of McClean & Hale [1940] and of Favilli [1940] indicates that the viscosity of vitreous humour preparation is decreased not only by the diffusing factors of biological origin, but also by some compounds known to increase the area of spread of indicators when tested for diffusing activity. The two types of compounds examined have been ascorbic acid and diazo compounds.

It has been established [Madinaveitia & Quibell, 1940] that, under certain conditions, the concentration of testicular extract or snake venom required to bring about a given change of the viscosity of a preparation of vitreous humour is inversely proportional to the time required for this change to take place. The effect of the concentration of ascorbic acid on the time required to decrease by a given amount the viscosity of a vitreous humour preparation does not follow such a simple law as testicular extracts of snake venom (Fig. 1). It is therefore unlikely that the mechanism of the reaction in which the viscosity is lost is the same in the case of ascorbic acid as in those of preparations from testicle or snake venom.

The spreading properties of diazobenzene-*p*-sulphonic acid and of some proteins coupled with it have been known for some time [Claude, 1933; 1935]. As reported by Favilli [1940] diazobenzene-*p*-sulphonic acid also causes a reduction in the viscosity of mucin preparations. The same effect has now been observed with diazotized aniline. Aniline, sulphanilic acid and sodium nitrite are without effect. When diazobenzenesulphonic acid is coupled with either dimethylaniline or with phenol the resulting dyestuff has no effect on the viscosity of vitreous humour preparations.

It is difficult to picture the type of reaction which occurs between the diazo compound and the viscous material which is of a polysaccharide nature, built up of acetyl glucosamine and uronic acid residues [Palmer *et al.* 1937]. Phenylhydrazine reacting with α-aminocarbonyl compounds will form osazones [Fischer, 1893], in some cases even when the nitrogen is substituted [Jacob & Madinaveitia, 1937]. Similarly it has been found in these Laboratories by Miss M. East that phenylhydrazine reacts with *N*-acetyl glucosamine yielding glucosazone. A slow reduction of the viscosity of vitreous humour preparations is observed when phenylhydrazine acts on them. In the reacting mixture the presence of diazo compounds can be detected. Since the reaction is greatly accelerated by H_2O_2 (Fig. 2) it seems likely that small amounts of diazobenzene formed by the oxidation of phenylhydrazine are responsible for the decrease in the viscosity. Neither semicarbazide nor hydroxylamine have any effect on the viscosity of vitreous humour preparations so that the action of phenylhydrazine cannot be due merely to its property of reacting with carbonyl groups.

[1] Beit Memorial Research Fellow.

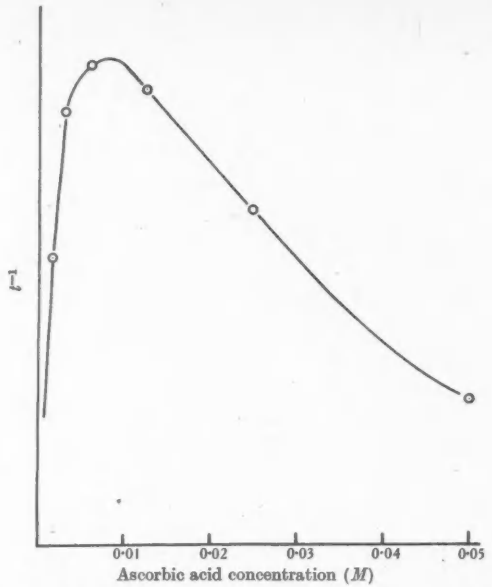
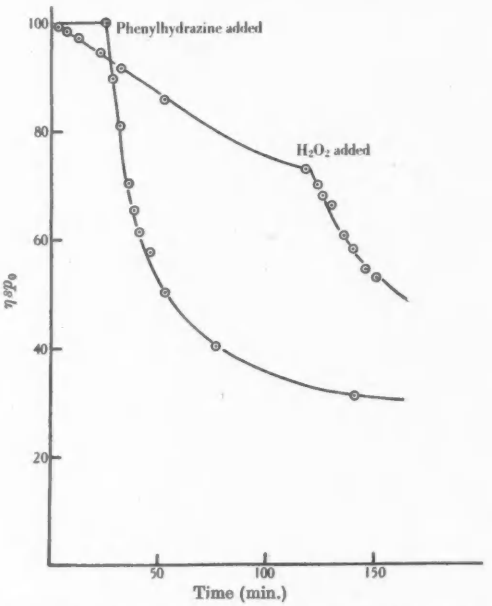


Fig. 1. Effect of the concentration of ascorbic acid on the rate of decrease of the viscosity of vitreous humour preparations.



Action of phenylhydrazine and H_2O_2 on the viscosity of vitreous humour.

Experimental

Action of ascorbic acid. Ascorbic acid (1·7 g.) dissolved in *M* citrate buffer *p*H 4·7 (15 ml.) was partially neutralized with *M* NaOH (5 ml.). Dilutions of the solution in *M* citrate buffer (1 ml.) were allowed to react with a solution of a vitreous humour acetone powder (4 ml.) in *M*/5 citrate buffer having *M* NaCl in a viscosimeter in a thermostat (40°). The technique used for following the fall of viscosity was that previously described [Madinaveitia & Quibell, 1940]. The results obtained are plotted in Fig. 1.

*Action of diazobenzene-*p*-sulphonic acid.* An ice-cold suspension of the diazo compound in water (2 ml.) was mixed with chilled *M* NaOH (2 ml.) and *M* citrate buffer *p*H 4·7 (2 ml.) was added to the mixture. This solution (1 ml.) was further diluted with citrate buffer which was *M*/2 in NaCl. A solution of a vitreous humour preparation (see above) (5 ml.) was allowed to react with the diluted solution of the diazo compound (1 ml.) at 25°.

Action of aniline and diazobenzene. A vitreous humour solution as used in the previous experiment (5 ml.) was mixed with *M*/5 aniline hydrochloride (0·5 ml.). The viscosity remained unchanged for 20 min., when *M*/5 $NaNO_2$ (1 ml.) was added. The viscosity now decreased at a speed comparable with that observed with diazobenzene-*p*-sulphonic acid.

Action of phenylhydrazine. Freshly distilled phenylhydrazine (1 ml.) was dissolved in *M* citrate buffer *p*H 4·7 (29 ml.). A sample of this solution (1 ml.) was allowed to react with a solution of vitreous humour acetone powder (5 ml.) in buffered *M* NaCl at 25°. The viscosity slowly decreased. After 1 hr. a small amount (0·05 ml.) was removed and acidified with HCl. On addition of a solution of α-naphthylamine in HCl a pink colour developed. A control test with phenylhydrazine solution alone was negative.

A buffered solution of vitreous humour (5 ml.) was mixed with the above phenylhydrazine solution (1 ml.). After the mixture had stood for 2 hr. at 25° H_2O_2 (0·1 ml. 30 %) was added. Now the viscosity rapidly decreased (Fig. 2). The same vitreous humour solution (5 ml.) was mixed with 30 % H_2O_2 (0·5 ml.). The viscosity of the mixture did not decrease with time, but when phenylhydrazine solution (1 ml.) was added the viscosity at once started to decrease (Fig. 2).

Summary

The observations of McClean & Hale [1940] and of Favilli [1940] that ascorbic acid and diazo compounds produce a gradual diminution of the viscosity of mucin preparations have been confirmed. The effect of the concentration of ascorbic acid on the time required to bring about a given decrease of the viscosity of vitreous humour preparations does not follow the same law as testicular extracts or snake venom. Phenylhydrazine has the effect of lowering the viscosity of mucins in a similar way. The action is much more marked in the presence of H_2O_2. Other carbonyl reagents are inactive.

REFERENCES

Claude (1933). *Science*, **78**, 151.
—— (1935). *J. exp. Med.* **62**, 229.
Favilli (1940). *Nature, Lond.*, **145**, 866.
Fischer (1893). *Ber. dtsch. chem. Ges.* **26**, 95.
Jacob & Madinaveitia (1937). *J. chem. Soc.* p. 1929.
Madinaveitia & Quibell (1940). *Biochem. J.* **34**, 65.
McClean & Hale (1940). *Nature, Lond.*, **145**, 866.
Palmer, Smith & Meyer (1937). *J. biol. Chem.* **119**, 491.

46. DIFFUSING FACTORS

9. THE EFFECT OF SALTS ON THE ACTION OF TESTICULAR EXTRACTS ON THE VISCOSITY OF VITREOUS HUMOUR PREPARATIONS

By J. MADINAVEITIA[1] and T. H. H. QUIBELL

From the Chemistry Department, University of Manchester

(*Received 10 March 1941*)

The reduction of the viscosity of certain mucins by testicular extracts and by mucinases from other sources of diffusing factors has been associated by Chain & Duthie [1939] with the characteristic spreading properties of these factors. Comparison of the relative diffusing and mucolytic activities of diffusing factor preparations [Madinaveitia *et al.* 1940] requires a relatively accurate quantitative estimation of the two effects. Methods are now available which permit such a comparison to be carried out; the diffusing activity can be determined with enough accuracy by the technique of Bacharach *et al.* [1940] and the available viscosimetric method [Madinaveitia & Quibell, 1940] accurately measures mucolytic activity. The results obtained by these methods do not quite agree with the view that the effect of diffusing factors is solely due to the mucolytic activity of the assayed preparations.

The viscosimetric method for the estimation of mucinases is based on the fact that the rate of decrease of the viscosity of vitreous humour preparation is proportional to the concentration of enzyme present in the reacting mixture, provided that the reaction is carried out under certain conditions. The mucolytic activities of a number of diffusing factor preparations have now been compared by this method. The results obtained are in good agreement when comparison is made at different concentrations of enzyme (c) after the original viscosity of the substrate (ηsp_0) has been reduced to a given value ($\%\eta sp_0$). The product of the concentration of enzyme and the time (t) required to reach the chosen viscosity level ($c \times t$) is practically constant for each preparation whatever is the decrease in the viscosity, provided that it is the same for all the samples compared (Table 1).

Table 1

		$\%\eta sp_0$			
		64·8	57·7	48·7	40·8
Preparation	c mg./6 ml.	$c \times t$			
Glaxo standard	0·49	122	196	343	538
	0·37	140	229	370	573
	0·245	134	238	368	587
Madinaveitia's [1938] standard	2·55	1120	1730	2670	4450
	1·915	950	1650	2680	4600
Crude *Crotalus atrox* venom	4·84	1350	2410	3950	5550
	2·41	1570	2340	3740	6620
Purified *Crotalus atrox* venom	1·08	194	335	540	840
	0·54	232	365	567	890
	0·36	254	397	630	950

[1] Beit Memorial Research Fellow.

Using one preparation as a standard of reference, the relative mucolytic activity of each of the others is the same irrespective of the viscosity level at which comparison is carried out (Table 2).

Table 2

Preparation	$\%\eta sp_0$ 71·5	63·5	45·0	35·0
	Ratio to Glaxo Standard			
From testicle:				
Glaxo standard	100	100	100	100
Madinaveitia standard	23·5	24·7	23·2	24·2
M/10 acetic acid extract	84	85	79	67?
$(NH_4)_2SO_4$-purified	150	152	147	147
From *Crotalus atrox*:				
Crude venom	19·9	17	15·7	15·9
$(NH_4)_2SO_4$-purified venom	51·5	57·5	67	69

All comparisons were carried out under the conditions originally suggested [Madinaveitia & Quibell, 1940], i.e. in 0·66 *M* NaCl and in the presence of 0·18 *M* citrate buffer *p*H 4·7. However, on testing by this method a mucinase preparation from *Clostridium welchii* prepared by Dr D. McClean of the Lister Institute we found that contrary to his indications it was practically inactive. This apparent discrepancy was due to the fact that Dr McClean's estimation of the mucolytic activity of this preparation had been carried out in the absence of NaCl. In view of this an investigation has now been carried out on the effect of salts on the reaction in which the viscosity of vitreous humour preparations is destroyed by the action of testicular extracts. A summary of the results has already been published [Madinaveitia & McClean, 1940]. The substrate used was prepared from an acetone powder of bovine vitreous humour (1 g.) by extraction with *M*/50 NaOH (50 ml.) and precipitation of the centrifuged and acidified (10 ml. *M* acetic acid) extract with 6 vol. acetone. After washing the precipitate with ether and drying *in vacuo* a white powder was obtained which was almost completely soluble in water but which still contained some salts. The source of enzyme was a preparation obtained by $(NH_4)_2SO_4$-fractionation from an acetic acid (2 l. *M*/10) extract of dry testicle powder (100 g.). The material precipitated between 30 and 70 % of saturation was redissolved, dialysed, subsequently electrodialysed until the conductivity was practically constant and finally dried whilst frozen. It was salt-free. The techniques used for estimating the viscosity and for determining the half-life time of the viscous substrate were those already described [Madinaveitia & Quibell, 1940].

As previously reported addition of salts has the effect of decreasing the specific viscosity (ηsp_0) of the substrate (Tables 3 and 5). However, the relationship between specific viscosity and the concentration of the substrate is the same at any given NaCl concentration as indicated by the fact that the ratio of the viscosity of the substrate (ηsp_0) to that of the substrate diluted with 1 vol. of the corresponding NaCl solution ($\eta sp_{\frac{1}{2}}$) is constant (Table 3). The only ratio which differs significantly from the others is that in which there is no NaCl present but this can be easily accounted for by the fact that the vitreous humour preparation used was not absolutely free of electrolytes. Reduction of the concentration of electrolyte on dilution with distilled water would render the more diluted substrate somewhat more viscous than expected. Electrodialysis throws the viscous materials out of solution.

When a series of samples of electrodialysed enzyme (0·5 mg. in 1 ml. water) are allowed to react with a substrate solution (5 ml. 2 %) in NaCl of increasing concentration the half-life time (t) of the viscous material decreases as the concentration of NaCl in the reacting mixture becomes larger and after reaching a minimum it increases again (Table 3). Therefore there is an optimum salt concentration for the activity (t^{-1}) of the enzyme.

Table 3

NaCl M	η^{sp}_0	η^{sp}_t	$\frac{\eta^{sp}_0}{\eta^{sp}_t}$	t min.	t^{-1}
0·00000	1·44	0·752	1·92	60	0·012
0·00083	1·28	0·596	2·14	30	0·033
0·00167	1·12	0·513	2·18	15·5	0·064
0·00334	0·94	0·443	2·13	12·5	0·080
0·00500	0·850	—	—	12·5	0·080
0·00667	0·775	—	—	9·0	0·112
0·00833	0·725	0·339	2·14	6·0	0·167
0·01677	0·593	0·279	2·13	4·2	0·238
0·0334	0·491	0·234	2·10	3·3	0·303
0·0500	0·441	—	—	2·3	0·425
0·0667	0·417	—	—	0·7	1·43
0·0833	0·400	—	—	0·7	1·43
0·167	0·339	—	—	0·7	1·43
0·334	0·308	—	—	3·0	0·333
0·500	0·289	—	—	3·2	0·312
0·667	0·283	—	—	4·7	0·213
0·833	0·270	—	—	14·5	0·069
1·670	0·259	—	—	30	0·033

The same effect was observed when a similar experiment was carried out in the presence of 0·2 M citrate buffer pH 4·7 and with concentrations of substrate and of enzyme different from those in the previous experiment. The same picture is obtained at whatever fraction of the original viscosity the comparison is carried out (Table 4). In addition to NaCl certain other salts in dilute solution

Table 4

NaCl M	$\%\eta^{sp}_0$					
	65	60	55	50	45	40
	Sec. required to reach $\%\eta^{sp}_0$					
0·00	300	380	900	1580	2400	3500
0·01	190	240	310	440	700	1050
0·02	160	240	350	530	880	1450
0·05	100	190	320	500	750	1050
0·10	130	210	370	550	820	1250
0·16	110	240	330	480	700	1050
0·21	130	240	370	550	820	1250
0·53	150	290	500	850	1320	2100
1·56	170	1050	1900	—	—	—
2·08	1300	2900	5000	—	—	—

also enhance the action of the enzyme and all the salts examined inhibit at relatively high concentrations. In Table 5 the effect of increasing amounts of NaCl is compared with that of other salts. The reaction was carried out in the presence of 0·2 M Na citrate buffer pH 4·7.

The method suggested [Madinaveitia & Quibell, 1940] for the comparison of the activities of mucinase preparations depends on the half-life time of the substrate being inversely proportional to the concentration of enzyme. This relationship does not hold good when working in an unbuffered solution at low

Table 5

	t	η_{sp_0}	t	η_{sp_0}	t	η_{sp_0}
Without salt	15	0·515	15	0·515	15	0·515
Molarity of salt	0·0083		0·083		0·83	
NaCl	7·5	0·430	7	0·392	29·5	0·330
KCl	7·5	0·435	7	0·402	29·5	0·326
NH_4Cl	5·5	0·472	5·5	0·458	45	0·310
NaI	11·5	0·404	13·5	0·382	100	0·325
NaBr	9·5	0·418	9·5	0·398	95	0·320
Na_2SO_4	15	0·422	40	0·407	200	0·450

NaCl concentrations. Under these conditions, when the concentration of enzyme is increased, the half-life time of the substrate decreases much more rapidly than expected (Fig. 1). This effect is most marked in the absence of NaCl and it becomes less noticeable as the concentration of salt increases. After a certain level of salt concentration has been reached the half-life time of the substrate becomes inversely proportional to the concentration of enzyme and thereafter this relationship is maintained although the reaction velocity decreases.

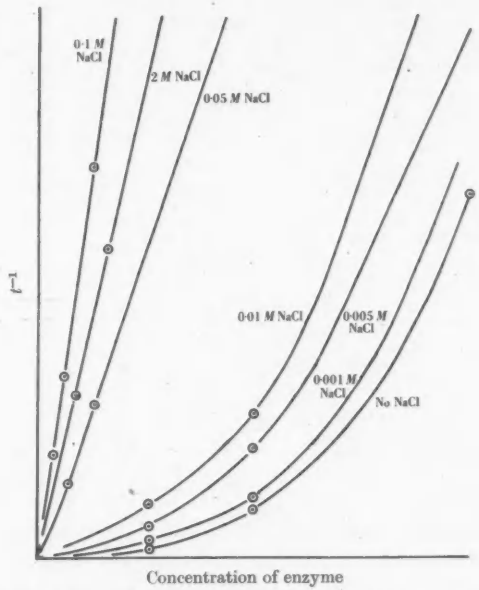


Fig. 1. Effect of the concentration of NaCl on the dose-response curve of testicular mucinase.

Testicular mucinase is not the only enzyme whose action is enhanced by the presence of salts. The effect of neutral salts on the action of different amylases is well known and has been studied in some detail [cf. Myrbäck, 1926; Sherman *et al.* 1928]. It has been found that some ions increase the activity of amylases, changing the *p*H optimum. An alteration of the kinetics of the reaction between amylase and starch due to the presence of NaCl, such as observed with testicular mucinase, has never been recorded as far as we are aware.

SUMMARY

The time required to reduce the viscosity of vitreous humour preparations to half its original value is inversely proportional to the concentration of testicular mucinase present in the reacting mixture, provided that the reaction is carried out in the presence of NaCl. At low concentrations of salt this time decreases very rapidly as the concentration of enzyme increases.

The authors' thanks are due to Prof. A. R. Todd for his continued interest and advice. They are also grateful to Dr D. McClean of the Lister Institute for supplies of bacterial filtrates and much useful discussion. Grants from Imperial Chemical Industries are gratefully acknowledged.

REFERENCES

Bacharach, Chance & Middleton (1940). *Biochem. J.* **34**, 1464.
Chain & Duthie (1939). *Nature, Lond.* **144**, 977.
Madinaveitia (1938). *Biochem. J.* **32**, 1470.
—— & McClean (1940). *J. Soc. Chem. Ind., Lond.* **59**, 418.
—— & Quibell (1940). *Biochem. J.* **34**, 625.
—— Todd, Bacharach & Chance (1940). *Nature, Lond.* **146**, 197.
Myrbäck (1926). *Hoppe-Seyl. Z.* **159**, 1.
Sherman, Caldwell & Adams (1928). *J. Amer. chem. Soc.* **50** 2535.

47. ESTERS OF PHOSPHORIC ACID
4. PHOSPHORYL HYDROXYAMINO-ACIDS

By R. H. A. PLIMMER

From the Chemistry Department, St Thomas's Hospital Medical School, London, S.E.

(*Received 12 March 1941*)

PHOSPHOTYROSINE was made by Levene & Schormüller [1933] by the action of $POCl_3$ in CCl_4 on formyltyrosine in presence of MgO. From the Mg salt of formylphosphotyrosine the formyl group was removed by hydrolysis with HCl and phosphotyrosine was obtained through the Ba and Pb salts.

Levene & Schormüller [1934, 1] tried three methods of preparing phospho-*dl*-serine of which only direct phosphorylation with H_3PO_4 and P_2O_5 was successful. The Ba salt was isolated. Later Levene & Schormüller [1934, 2] prepared larger quantities and separated the stereoisomers by fractional crystallization of the brucine salts. They also isolated the Ba salt of phospho-*l*-hydroxyproline after direct phosphorylation. Phosphohydroxyaspartic acid and phosphohydroxyglutamic acid could not be made. Neither phosphoserine nor phosphohydroxyproline were prepared and very little information was given of the properties of the compounds. Further information was desirable, especially concerning the hydrolysis of these esters by acid, alkali and phosphatase to compare with caseinogen, caseo-phosphopeptone and other esters of phosphoric acid.

EXPERIMENTAL

1. *Phosphotyrosine*

The successful phosphorylation of amino-ethanol and choline by Plimmer & Burch [1937] by heating the substances with H_3PO_4 and P_2O_5 led to a trial with tyrosine. On direct heating, the mixture darkened and effervesced, but it was possible to isolate a small yield of the Ca salt of phosphotyrosine. Subsequent experiments were made by heating the mixture (2–5 g. tyrosine, 14–35 g. H_3PO_4 and 2–5 g. P_2O_5) in a small flask with $CaCl_2$ tube on a water bath for 1, 6, 48, 64 hr. to ascertain the condition for the best yield. The resulting syrup was dissolved in water and made to 500 ml. and an aliquot was taken for N determination to give information of the amount of substance in later solutions. The solution was made alkaline to phenolphthalein with a fine suspension of $Ca(OH)_2$, or with $Ba(OH)_2$ solution, and filtered from $Ca_3(PO_4)_2$ and excess $Ca(OH)_2$, or $Ba_3(PO_4)_2$, which was washed once or more with water until all the N was in solution. In one experiment the Ca salt was isolated by concentrating *in vacuo* and adding an equal volume of absolute alcohol. The air-dried salt was heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 4·48; P, 10·02; Ca, 13·55; H_2O, 14·53 %. $C_9H_{10}O_6NPCa$ requires N, 4·68; P, 10·36; Ca, 13·40; $+3H_2O$ requires H_2O, 15·30 %.) The low water content is probably due to diffculty in removing water from phosphoric esters. Some $CaCO_3$ was present.

In another experiment the Ba salt was isolated by adding an equal volume of absolute alcohol to the concentrated filtrate. It came down as a very fine precipitate; if kept in air it became syrupy and set to a glass which was easily

powdered. It was redissolved in water, reprecipitated and dried in a desiccator. For analysis, it was heated at 100° *in vacuo* over P_2O_5. (Found: N, 3·58; P, 7·48; Ba, 35·22; H_2O, 13·22 %. $C_9H_{10}O_6NPBa$ requires N, 3·53; P, 7·79; Ba, 34·65; $+4H_2O$ requires H_2O, 15·38 %.)

Again, the difficulty in removing H_2O from phosphoric esters will account for the low H_2O value. $BaCO_3$ was also present.

In other experiments lead acetate solution was added to the filtrate until there was no further precipitation. The Pb salt, after washing, was suspended in water and decomposed with H_2S and the filtrate from PbS concentrated and treated with an equal volume of absolute alcohol. The resulting crystals were filtered off and washed with 50 % alcohol. Further crops of phosphotyrosine were obtained on concentrating the solution and adding alcohol. The crops crystallizing out first consisted of nearly pure phosphotyrosine; later crops were admixed with tyrosine. The pure compound was obtained by redissolving, filtering and adding alcohol. The several preparations dried at 100° *in vacuo* over P_2O_5 gave on analysis: N, 5·32, 5·42, 5·41, 5·20; P, 11·92, 12·13, 11·89, 11·68 %. $C_9H_{12}O_6NP$ requires N, 5·34 %, P, 11·83 %.

Phosphotyrosine crystallizes in shining platelets; on heating it shrinks at 224° and melts at 225°. It dissolves slowly in cold water, more rapidly on warming. 0·2584 g. in 25 ml. 2*N* HCl gave in a 2 dm. tube a rotation of −0·19°, $[\alpha]_D - 9·19°$. These data do not agree with those of Levene & Schormüller [1933] who gave M.P. 253° and $[\alpha]_D - 2·0°$.

A 0·6 % solution of phosphotyrosine in water gave precipitates only with Pb acetate and $HgNO_3$ solutions. The other salts are thus soluble in water. The Pb salt, isolated in one preparation, dried in air and heated at 100° *in vacuo* over P_2O_5, gave on analysis: N, 2·93; P, 6·86; Pb, 43·74; H_2O, 3·88 %. $C_9H_{10}O_6NPPb$ requires N, 3·00; P, 6·65; Pb, 44·42; $+H_2O$ requires H_2O, 3·72 %.

Phosphotyrosine does not give a blue colour with Folin's phenol reagent. With Millon's reagent at room temperature it turns brown on standing; on warming there is slow development of colour, probably due to hydrolysis to tyrosine.

The yields of phosphotyrosine were 28 % on heating for 1 hr.
41 % ,, 6 hr.
48 % ,, 48 hr.
55 % ,, 64 hr.

the extra time thus being advantageous.

2. *Phosphohydroxyproline*

l-Hydroxyproline (Roche) was phosphorylated in the same way as tyrosine with 2 g. at a time. The syrupy residue was dissolved in water, and the solution treated with $Ba(OH)_2$ solution, or $Ca(OH)_2$ suspension. The filtrate and washings from the precipitate were concentrated *in vacuo* and precipitated with alcohol. The yield of Ba salt was 48 % on phosphorylating for 24 hr. and 51 % after 40 hr. At room temperature the phosphorylation was 26 % in 13 days. The Ba salt was dried in air and heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 3·46; P, 8·81; Ba, 39·23; H_2O, 16·24 %. $C_5H_8O_6NPBa$ requires N, 4·05; P, 8·96; Ba, 39·50; $+4H_2O$ requires H_2O, 17·23 %.)

If precipitated from hot solution, H_2O was 3·50 to 3·88 %, and the salt dried at 100° *in vacuo* over P_2O_5 showed N 3·9, P 8·5, Ba 36·6 %, indicating $1H_2O$ and partial conversion into acid salt.

The Pb salt was prepared from the Ba or Ca salt by precipitation with Pb acetate. After filtration and washing it was suspended in water and decomposed with H_2S. The filtrate from PbS was evaporated *in vacuo* to a small volume and alcohol added in small portions. Phosphohydroxyproline came down as an oil, which could be hardened with alcohol and became crystalline, or it could be obtained crystalline directly by very slowly adding alcohol and scratching.

The analyses of the several specimens of phosphohydroxyproline presented difficulty in the interpretation of the results:

On heating the air-dried substance at 100° *in vacuo* over P_2O_5 and analysing the residue:

H_2O varied from 4·96 to 5·75 %;

N varied from 5·81 to 6·11 %; average 6·00 %;

P varied from 13·46 to 14·00 %; average 13·70 %.

The most usual figures were N, 6·10; P, 13·58 %.

$C_5H_{10}O_6NP$ requires N, 6·63; P, 14·69 %. $C_5H_{10}O_6NP$, H_2O requires N, 6·11; P, 13·54; H_2O, 7·86 %.

The air-dried substance showed N, 5·66; P, 13·28 %.

$C_5H_{10}O_6NP$, $1{\cdot}5H_2O$ requires N, 5·88; P, 13·03 %.

The loss of H_2O on heating at 100° is thus between 1·5 and $1H_2O$.

Not until the substance was heated to 130° *in vacuo* over P_2O_5 was the water of crystallization completely driven off. (Found: H_2O, 10·91; N, 6·66; P, 14·96 %. On exposing the dried substance to air it took up H_2O, 7·71 %.)

The air-dried substance would thus be $C_5H_{10}O_6NP$, $1{\cdot}5H_2O$; after heating to 100° $C_5H_{10}O_6NP$, H_2O; after heating to 130° $C_5H_{10}O_6NP$.

Phosphohydroxyproline crystallizes in fine needles, M.P. 115°. The anhydrous substance melts at 130–131° with frothing but without decomposition. 0·2738 g. dissolved in 15 ml. H_2O (1·825 %) in a 2 dm. tube gave a rotation of −1·05°, $[\alpha]_D$ −28·76°. The solution gave only an insoluble lead salt.

3. *Phosphoserine*

Poor yields of the Ba salt of phosphoserine were obtained by Levene & Schormüller [1934, 1, 2] by the interaction of serine, H_3PO_4 and P_2O_5 for 40 hr. at room temperature; thus 1·85 g. from 4 g. serine = approx. 15 %, and 106 g. from 200 g. serine = approx. 17 %.

As good yields of phosphotyrosine and phosphohydroxyproline were obtained on heating at 100°, the same procedure of heating 1 g. serine, 7 g. H_3PO_4 and 1 g. P_2O_5 was tried. There was no product either on heating for 24 or 45 hr. On keeping the mixture for long periods of 4, 24 and 46 days yields of Ba salt amounting to 0·5, 16 and 26 % resulted. It was possible therefore that any phosphoserine formed at 100° was hydrolysed.

Heating in an autoclave at 20 lb. pressure for 1–1·5 hr. gave yields of Ba salt varying from 8 to 21 %, no improvement on the yields at room temperature. An advantage is that the preparation takes less time.

The isolation of the Ba or Ca salt was effected in the usual way. The specimens of Ba and Ca salts after drying in air were heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 4·27; P, 9·63; Ba, 42·62; H_2O, 14·69 %. $C_3H_6O_6NPBa$ requires N, 4·37; P, 9·67; Ba, 42·87; $+3H_2O$ requires H_2O, 14·43 %.)

Other preparations contained $BaCO_3$.

The Ba salt of serine could be recovered from the preparations in which there was no phosphorylation and also from the filtrates from Ba phosphoserine. This salt is less soluble in water than expected.

The Pb salt of phosphoserine was precipitated on adding basic Pb acetate to the solution of the Ba salt. The air-dried salt was heated to 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 1·86; P, 4·39; Pb, 61·91; H_2O, 6·09 %. It appears to be a basic salt: $C_3H_6O_6NPPb$. $Pb(OH)_2$ requires N, 2·22; P, 4·91; Pb, 65·61; $+3H_2O$ requires H_2O, 7·88 %.)

Phosphoserine was prepared from the Pb salt in the usual way by precipitating with 3 to 4 vol. alcohol. Only three specimens, amounting to 0·2, 0·14 and 0·3 g., were made. The air-dried substances heated at 100° *in vacuo* over P_2O_5 gave N, 7·94, 7·56, 7·37; P, 16·72, 16·83, 16·81 %. $C_3H_8O_6NP$ requires N, 7·57; P, 16·76 %.

Phosphoserine crystallizes in irregular platelets which darken on heating and melt at 165–166° with decomposition. A 0·5 % solution gave insoluble salts only with Pb acetate and $HgNO_3$.

4. *Phosphoisoserine*

Phosphorylation of *iso*serine, prepared by Burch [1930], did not take place on standing at room temperature for 54 days. Poor yields (13–16 %) resulted on heating 1 g. *iso*serine, 7 g. H_3PO_4 and 1 g. P_2O_5 at 20 lb. pressure for 1–1·5 hr. The isolation was as described under phosphoserine.

The Ba salt could not be obtained pure and with the small amount available conversion into phospho*iso*serine could not be attempted.

5. *Phosphothreonine*

The mixture of stereoisomers of β-hydroxy-α-aminobutyric acid was prepared by the method of West & Carter [1937]. A yield of 89 % of the mercuric acetate compound of β-methoxybutyric acid was obtained, and from this a 92·5 % yield of β-methoxy-α-bromobutyric acid. 10 g. portions were converted into β-methoxy-α-aminobutyric acid by heating with conc. NH_4OH in a pressure bottle in boiling water for 1·5 hr. The yields were 60–63 %. Hydrolysis with HBr gave yields of 60–68 % of β-hydroxy-α-aminobutyric acids. The pure compound, recrystallized from water and alcohol, had N, 11·74 % (calc. 11·76 %) and M.P. 225–226° with decomposition, which corresponds with 228–229° given by Adkins & Reeve [1938] for *dl*-threonine, as compared with 252–253° for *dl-allo*threonine.

In comparison with serine and *iso*serine threonine is readily converted into phosphothreonine. 2 g. portions were heated with 10 g. H_3PO_4 and 2 g. P_2O_5 on a water bath for 5–20 hr. The yield of Ba salt averaged 46 %. The air-dried salt heated at 100° *in vacuo* over P_2O_5 gave N, 3·93; P, 8·94; Ba, 42·06; H_2O, 9·56 %. $C_4H_8O_6NPBa$ requires N, 4·19; P, 9·26; Ba, 41·07; $+2H_2O$ requires H_2O, 9·73 %.

The Pb salt was air-dried and heated at 100° *in vacuo* over P_2O_5 for analysis. (Found: N, 2·97; P, 7·22; Pb, 51·74; H_2O, 10·10 %. $C_4H_8O_6NPPb$ requires N, 3·46; P, 7·67; Pb, 51·24; $+3H_2O$ requires H_2O, 11·84 %.)

Phosphothreonine prepared from the Pb salt by adding 2 vol. alcohol and recrystallizing was dried at 100° *in vacuo* over P_2O_5. (Found: N, 6·37; P, 14·24; H_2O, 3·10 %. $C_4H_{10}O_6NP$ requires N, 7·04; P, 15·56 %. $C_4H_{10}O_6NP,H_2O$ requires N, 6·45; P, 14·27; H_2O, 8·34 %.)

More H_2O was driven off at 115°, but the substance turned brown and partially decomposed.

Phosphothreonine crystallizes from water and alcohol in small square plates, massing in cubes on slow crystallization, M.P. 169° with decomposition. A 0·5 % solution gave insoluble salts only with Pb acetate and $HgNO_3$.

6. *Phosphohydroxyaspartic acid*

Hydroxyaspartic acid, prepared by Burch [1930], could not be phosphorylated either at room temperature, at 100° or at 20 lb. pressure.

Hydrolysis of the esters

Phosphotyrosine was used itself. In the other cases solutions of the Na salts were prepared from the Ba salts by decomposition with the calculated quantity of Na_2SO_4 and filtration from $BaSO_4$.

A. HCl. About 100 ml. of solution were prepared. 1 ml. was taken for estimation of total P and 10 ml. portions for inorg. P by precipitation with NH_4Mg citrate. The NH_4MgPO_4 was filtered off, washed with NH_4OH, dissolved in 2 *N* HNO_3 and the P estimated by the micro-method of Plimmer [1933]. The results were as follows:

1. *Phosphotyrosine*

By *N* HCl

0·8 g. in 105 ml. *N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
48 ,,	0·31	3·2
5 hr. at 100°	4·79	50·0
21 ,,	8·32	86·8
28 ,,	8·55	89·2
48 ,,	8·49	88·6
72 ,,	9·41	98·2
Total P	9·58	

2. *Phosphohydroxyproline*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
4 ,,	0·33	12·4
8 ,,	0·55	20·7
24 ,,	1·18	44·3
32 ,,	1·36	51·1
48 ,,	1·66	62·4
72 ,,	1·82	68·4
Total P	2·66	

3. *Phosphoserine*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
24 hr. at 100°	1·23	60·6
48 ,,	1·49	73·4
72 ,,	1·69	83·2
Total P	2·05	

4. *Phosphoisoserine*

By *N*/2 HCl

50 ml. Na salt + 50 ml. *N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
42 ,,	0	0
8 hr. at 100°	1·02	44·0
24 ,,	1·95	84·0
48 ,,	1·96	84·5
72 ,,	2·00	86·2
Total P	2·32	

5. *Phosphothreonine*

By *N* HCl

50 ml. Na salt + 50 ml. 2*N* HCl

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
6 hr. at 100°	0·89	18·3
12 ,,	1·83	37·6
18 ,,	2·50	51·3
24 ,,	3·04	62·4
48 ,,	4·26	87·5
Total P	4·87	

At 37° there was no hydrolysis by *N* HCl, except of phosphotyrosine which amounted to 3·2 % in 48 hr. At 100° all the esters were hydrolysed fairly rapidly.

B. NaOH. The samples were acidified with HCl before precipitation with NH_4Mg citrate. Silica from glassware caused trouble in filtration and difficulty in washing. It may retain some unhydrolysed ester, which will be subsequently hydrolysed by HNO_3.

1. *Phosphotyrosine*

By *N* NaOH

1 g. in 50 ml. H_2O + 50 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
48 ,,	0·03	0·4
5 hr. at 100°	0·07	1·0
24 ,,	0·25	3·7
48 ,,	0·28	4·2
72 ,,	0·35	5·2
Total P	6·70	

3. *Phosphoserine*

(1) By *N*/2 NaOH

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
6 ,,	0	0
25 ,,	0	0
24 hr. at 100°	0·90	50·9
48 ,,	1·24	70·0
Total P	1·77	

(2) By *N*/4 NaOH

50 ml. Na salt + 50 ml. *N*/2 NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
4 ,,	0	0
8 ,,	0·07	3·7
24 ,,	1·23	65·4
48 ,,	1·24	66·0
72 ,,	1·20	64·9
Total P	1·88	

(3) By *N*/2 NaOH in Cu flask

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
2 ,,	0·05	2·8
4 ,,	1·27	71·7
8 ,,	1·32	74·6
24 ,,	1·26	71·2
Total P	1·77	

2. *Phosphohydroxyproline*

(1) By *N* NaOH

52 ml. Na salt + 52 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
6 ,,	0	0
24 ,,	0·06	2·3
Total P	2·66	

The inorg. P seemed to be from adsorption on silica from glass vessel. Hydrolysis repeated in Cu flask.

(2) By *N*/2 NaOH

50 ml. phosphohydroxyproline + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 100°	0	0
3 ,,	0·06	1·8
6 ,,	0·03	0·9
24 ,,	0·34	10·1
48 ,,	0·07	2·1
72 ,,	1·13	33·7
120 ,,	1·27	37·9
Total P	3·35	

Hydrolysis apparently occurred after 24 hr. The low P figure at 48 hr. is probably an error.

4. *Phosphoisoserine*

By *N*/2 NaOH

50 ml. Na salt + 50 ml. *N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
40 ,,	0	0
24 hr. at 100°	0	0
48 ,,	0	0
72 ,,	0	0
Total P	2·43	

5. *Phosphothreonine*

By *N* NaOH

50 ml. Na salt + 50 ml. 2*N* NaOH

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	0	0
48 ,,	0	0
6 hr. at 100°	1·08	21·7
12 ,,	1·82	36·5
37 ,,	2·98	59·8
61 ,,	3·77	75·7
85 ,,	4·16	83·5
Total P	4·98	

All the esters are stable to NaOH at 37°. The stability contrasts with the instability of the phosphoric acid groups in caseinogen and caseo-phosphopeptone, which are completely hydrolysed by $N/4$ NaOH in 24 hr. At 100° there is only a small hydrolysis of phosphotyrosine by $2N$ NaOH in 72 hr.; this contrasts with the complete hydrolysis of phenyl phosphate [Plimmer & Burch, 1929]. Phosphohydroxyproline is only appreciably hydrolysed after 48 hr. Phosphoserine is hydrolysed to the extent of 60–70 % in 24 hr., but phospho*iso*serine is not hydrolysed. Phosphothreonine is hydrolysed like phosphoserine.

C. Kidney phosphatase. The enzyme solution was prepared by grinding minced rabbits' kidneys with sand and extracting with water + toluene for 24–48 hr. The extract was strained through muslin. Control experiments with boiled extract were made at the same time. Inorganic P was estimated after acidifying the solutions with HCl and precipitation with NH_4Mg citrate + NH_4OH. The procedure was then as before. The filtration of the solutions was very slow owing to precipitation of protein. The inorganic P of the control was deducted from that of the enzyme solution to give the change in P content due to hydrolysis.

1. *Phosphotyrosine*

25 ml. phosphotyrosine soln. + 40 ml. extract. *p*H 4

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0·32	6·0
24 ,,	2·59	49·1
48 ,,	3·42	64·9
96 ,,	3·62	68·7
Total P	5·27	

2. *Phosphohydroxyproline*

50 ml. Na salt + 50 ml. extract. *p*H 5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
4 ,,	0	0
7 ,,	0·08	2·9
23 ,,	0·82	29·7
30 ,,	1·00	36·2
48 ,,	1·57	56·9
72 ,,	2·10	76·1
96 ,,	2·07	75·0
Total P	2·76	

3. *Phosphoserine*

50 ml. Na salt + 50 ml. extract. *p*H 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
6 ,,	0·06	3·3
24 ,,	1·22	67·7
54 ,,	1·41	78·3
74 ,,	1·35	75·0
120 ,,	1·35	75·0
Total P	1·80	

4. *Phosphoisoserine*

50 ml. Na salt + 50 ml. extract. *p*H 7

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
5 ,,	8·5	45·2
24 ,,	10·2	54·2
48 ,,	11·6	61·1
72 ,,	10·8	57·4
96 ,,	11·1	59·0
Total P	18·8	

5. *Phosphothreonine*

50 ml. Na salt + 50 ml. extract. *p*H 7

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
5 ,,	0·40	10·8
24 ,,	1·80	48·6
29 ,,	2·11	57·0
48 ,,	2·60	70·3
72 ,,	3·09	83·5
Total P	3·70	

D. Intestinal phosphatase. The solution of enzyme was prepared from the mucous membranes of rabbits' intestines which were ground up with sand and extracted with water + toluene. Before use the extract was strained through

muslin. The experiments were made as with kidney extracts. Filtration of the solutions was very slow owing to precipitation of proteins.

1. *Phosphotyrosine*

25 ml. phosphotyrosine soln. +40 ml. extract. *p*H 4

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	1·64	29·8
24 ,,	3·56	64·7
48 ,,	3·69	67·1
96 ,,	3·69	67·1
Total P	5·50	

2. *Phosphohydroxyproline*

50 ml. Na salt +50 ml. extract. *p*H 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
3 ,,	1·05	59·0
7 ,,	1·14	64·0
22 ,,	1·23	69·1
46 ,,	1·18	66·3
72 ,,	1·37	77·0
96 ,,	1·30	73·0
Total P	1·78	

3. *Phosphoserine*

50 ml. Na salt +50 ml. extract. *p*H 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
24 ,,	+	+
48 ,,	+	+
72 ,,	0·68	30·2
120 ,,	1·37	60·9
168 ,,	1·51	67·1
Total P	2·25	

+ error in determinations.

4. *Phosphoisoserine*

50 ml. Na salt +50 ml. extract. *p*H 6·5

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
8 ,,	1·24	60·2
24 ,,	1·29	62·6
48 ,,	1·27	61·3
72 ,,	1·27	61·3
96 ,,	1·33	65·5
Total P	2·06	

5. *Phosphothreonine*

50 ml. Na salt +50 ml. extract. *p*H 8

	Inorg. P mg.	Hydrolysis %
After 0 hr. at 37°	0	0
5 ,,	0·11	3·0
24 ,,	0·51	13·6
29 ,,	0·60	16·0
48 ,,	0·93	24·7
72 ,,	1·09	29·0
Total P	3·76	

Slow hydrolysis probably due to alkaline reaction.

All the esters are hydrolysed by kidney and intestinal phosphatases. The hydrolyses were never complete even after long periods of action. Comparison of the rates was not made as extracts made at different times were used.

Summary

Phosphotyrosine, phosphohydroxyproline, phosphothreonine, phosphoserine and phospho*iso*serine have been prepared by phosphorylation of the amino-acids with $H_3PO_4 + P_2O_5$; in the case of phospho*iso*serine only the Ba salt was isolated. Phosphohydroxyaspartic acid could not be made in this way. The lead salts of all are insoluble in water, as also the mercurous salts except that of phosphohydroxyproline.

All the esters are hydrolysed by *N* HCl at 100°. All are stable to hydrolysis by *N*/2 or even *N* NaOH at 37°, but all except phospho*iso*serine are hydrolysed at 100°, phosphoserine and phosphothreonine fairly rapidly. All are hydrolysed by phosphatases of kidney and intestine.

REFERENCES

Adkins & Reeve (1938). *J. Amer. chem. Soc.* **60**, 1328.
Burch (1930). *J. chem. Soc.* p. 310.
Levene & Schormüller (1933). *J. biol. Chem.* **100**, 583.
—— —— (1934, 1). *J. biol. Chem.* **105**, 547.
—— —— (1934, 2). *J. biol. Chem.* **106**, 595.
Plimmer (1933). *Biochem. J.* **27**, 1810.
—— & Burch (1929). *J. chem. Soc.* p. 279.
—— —— (1937). *Biochem. J.* **31**, 398.
West & Carter (1937). *J. biol. Chem.* **119**, 103.

48. ON THE *p*H AT THE SURFACE OF OVALBUMIN MOLECULES, AND THE PROTEIN ERROR WITH INDICATORS

By JAMES FREDERIC DANIELLI[1]

From the Biochemical Laboratory, Cambridge

(*Received 12 March 1941*)

The *p*H in a surface phase is frequently different from that in a bulk phase in equilibrium with the surface. The surface *p*H may be calculated from the Gibbs-Donnan equilibrium [Danielli, 1937] using the equation

$$\frac{(\alpha_{Na})_s}{(\alpha_{Na})_b}=\frac{(\alpha_H)_s}{(\alpha_H)_b}, \tag{1}$$

where α denotes activity and suffixes s and b denote surface and bulk phases. If the various ionic species considered in any one phase all have the same activity coefficient, or if a given ion has the same activity coefficient in both phases, then the activity coefficients will cancel, giving

$$\frac{[Na]_s}{[Na]_b}=\frac{[H]_s}{[H]_b}, \tag{2}$$

where the square brackets refer to concentrations. Or, taking logarithms, and using the suffix D to denote this method of calculation,

$$(pH_s)_D-pH_b=-\log\frac{[Na]_s}{[Na]_b}. \tag{3}$$

If we had considered a diffusible anion instead of a cation, e.g. Cl^- instead of Na^+, we should have

$$(pH_s)_D-pH_b=+\log\frac{[Cl]_s}{[Cl]_b}. \tag{4}$$

Equation (3) or (4) can thus be taken as one method of defining the difference between pH_s and pH_b. Harvey & Danielli [1938] pointed out that these equations predicted that the *p*H at the surface of protein molecules is different from the bulk *p*H.

Linderstrøm-Lang [1924] pointed out that the concentration of ions at the surface of a colloidal particle is different from that in the surrounding bulk phases, and that the concentration ratio will be determined by the electrical potential between the surface of the colloid and the bulk phase. Linderstrøm-Lang predicted that the titration curves of proteins would be straight lines in the region of the iso-ionic point, and that the addition of salt would change the slope of these lines. This prediction was confirmed experimentally by Linderstrøm-Lang & Lund [1926]. But Linderstrøm-Lang did not calculate the actual *p*H at the surface of a colloidal particle.

Hartley & Roe [1940] have shown that pH_s may also be calculated from the electrokinetic potential ζ, using the equation

$$(pH_s)_E-pH_b=\frac{\zeta}{60}=0{\cdot}325u \tag{5}$$

for small particles at 25°. u is the mobility in μ/sec./volt/cm.

[1] Beit Memorial Medical Research Fellow.

Both methods of calculating $p\mathrm{H}_s$ give the average value of $p\mathrm{H}_s$ in the surface phase. Actually variation in $p\mathrm{H}_s$ must occur within the surface phase, as a function of distance from the charged groups of the surface. There is some theoretical difference between $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$, since $(p\mathrm{H}_s)_D$ can, strictly speaking, give only the *activity* of the H^+ ion in the surface phase, and $(p\mathrm{H}_s)_E$ can only give the *concentration* of H^+ in the surface phase.

This paper deals with (1) the calculation of $(p\mathrm{H}_s)_D$ and $(p\mathrm{H}_s)_E$ at the surface of ovalbumin molecules, (2) the correction of titration curves for the difference between bulk and surface *p*H, (3) the nature of the 'protein' error with indicators.

(1) *The p*H *at the surface of ovalbumin molecules*

Fig. 1 shows results for ovalbumin. $[(p\mathrm{H}_s)_E - p\mathrm{H}_b]$ is plotted against $p\mathrm{H}_b$, using the mobility measurements of Abramson [1934]. On the acid side of the isoelectric point the *p*H at the surface of the protein is greater than in bulk and on the alkaline side is less than in bulk.

Calculation of $(p\mathrm{H}_s)_D$ is more complicated. We need to know the molar concentration of Na^+ or Cl^- in the surface phase of the protein. We can obtain an approximate value (to be corrected later) by assuming that, when the protein is ionized as an acid, no Cl^- enters the surface phase, and that when ionized as a base no Na^+ enters the surface phase. Then the number of ions in the surface phase of one protein molecule will be approximately equal to the valency z of the protein molecule. The valency can be obtained from the titration curve. The theory of Debye & Hückel enables us to calculate the thickness δ of the surface phase. Then the volume of the surface phase is $V = \frac{4}{3}\pi[(r+\delta)^3 - r^3]$, where r = radius of the protein molecule. For ovalbumin $r = 2{\cdot}75 \times 10^{-7}$ cm. [Adair & Adair, 1940] and in $N/100$ uni-univalent electrolyte $\delta = 3{\cdot}1 \times 10^{-7}$ cm. Hence $V = 7{\cdot}5 \times 10^{-19}$ ml. per molecule. Then the concentration of ions in the surface phase is

$$\frac{z \times 1000}{V \times 6{\cdot}06 \times 10^{23}} = 2{\cdot}2z \times 10^{-3} \text{ g. mol./l.} \qquad (6)$$

This assumes that none of the mobile ions neutralizing the fixed charges on the protein surface can penetrate into the interior of the protein molecule. Having obtained this first approximation to the concentration in the surface, we can now use the Gibbs-Donnan equation:

$$\frac{[\mathrm{Na}]_s}{[\mathrm{Na}]_b} = \frac{[\mathrm{Cl}]_b}{[\mathrm{Cl}]_s} = \text{constant} \qquad (7)$$

and the condition for electrical neutrality in the interface:

$$[\mathrm{Na}]_s + [\mathrm{Cl}]_s + [\mathrm{Pr}]_s = 0 \qquad (8)$$

to calculate the true value of $[\mathrm{Na}]_s$ by successive approximations. $[\mathrm{Pr}]_s$ = concentration of ions due to groups fixed on the protein; e.g. for the case where $z = 10$, the protein is an anion and the bulk phase is $N/100$ NaCl, we have as a first approximation $[\mathrm{Na}]_s = 10 \times 2{\cdot}2 \times 10^{-3} = 0{\cdot}022$. Then from equation (7):

$$\frac{0{\cdot}022}{0{\cdot}01} = \frac{0{\cdot}01}{[\mathrm{Cl}]_s}$$

or

$$[\mathrm{Cl}]_s = 0{\cdot}0045.$$

Hence a better approximation to $[\mathrm{Na}]_s$ is $0{\cdot}022 + 0{\cdot}0045 = 0{\cdot}0265$. Then this value of $[\mathrm{Na}]_s$ can be substituted in (7) to get a more accurate value of $[\mathrm{Cl}]_s$. By a series of such successive approximations we obtain values for $[\mathrm{Cl}]_s$ and $[\mathrm{Na}]_s$ which are accurate. Having obtained $[\mathrm{Na}]_s$, and knowing $[\mathrm{Na}]_b$ and $p\mathrm{H}_b$,

we can now calculate $(pH_s)_D$ from equation (3). The results of such calculations are shown in Fig. 1.

In the above calculation there are three sources of error, whose magnitude may be estimated. (1) The possibility of some of the mobile ions in the surface phase being inside the micelle has been neglected. Suppose that the mobile ions can penetrate readily into the whole of the protein molecule—this will be the greatest possible degree of penetration. Then we must regard as the volume in which the mobile ions are distributed not only the external layer, given by the Debye-Hückel theory, but also a layer inside the molecule of the same thickness, i.e. $3{\cdot}1 \times 10^{-7}$ cm. in $N/100$ solutions. This is greater than the radius of the ovalbumin molecule and so in practice we must simply add to the previously obtained value of V the volume of the ovalbumin molecule, obtaining

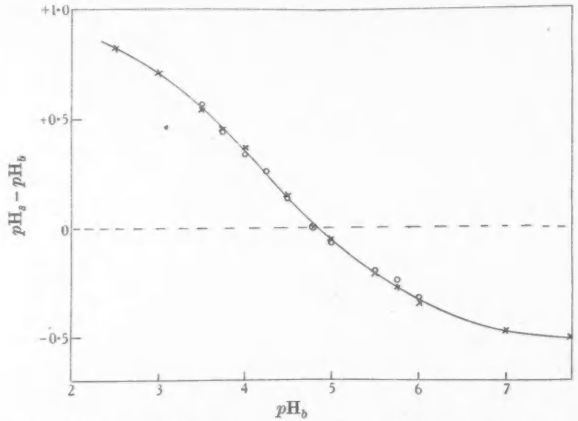


Fig. 1. Values of $(pH_s - pH_b)$ plotted against pH_b. × Calculated from Gibbs-Donnan equilibrium; o calculated from electrokinetic mobility.

$V' = 8{\cdot}37 \times 10^{-19}$ ml. per molecule instead of $7{\cdot}5 \times 10^{-19}$ ml. as the volume in which the z mobile ions are distributed. Using V' instead of V in equation (6) will lead to a reduction in $[(pH_s)_D - pH_b]$ of not more than 0·04 pH units. This is the maximum possible error introduced by ignoring penetration of ions into the interior of the ovalbumin molecule.

(2) The values of z have been taken from the results of Kekwick & Cannan [1936] for salt-free solutions of ovalbumin. In 0·01 M NaCl this value may be up to 10 % too low [Cannan, 1938]. Hence the value of z used in equation (6) may be too low by this amount, and $(pH_s)_D - pH_b$ will be too low by not more than 0·04 pH unit. Errors (2) and (3) are of opposite sign.

(3) Adsorption of ions by processes other than those considered here, i.e. specific adsorption, would also introduce an error. The results of Adair & Adair [1940] suggest that this effect is quite small for ovalbumin.

From this discussion it follows that these errors may lead to the values of $[(pH_s)_D - pH_b]$ given in Fig. 1 being in error by a factor of not more than $\pm 0{\cdot}04$ pH unit. We may therefore conclude that the values of the pH at the surface of ovalbumin molecules in 0·01 M NaCl solution, calculated by the two methods of equation (3) and equation (5), are in approximate agreement, since the points calculated by the two methods fall approximately in the same place.

(2) *The influence of ionic strength on surface* p*H and on the titration curve of ovalbumin*

We may write equation (4), for molecules on the acid side of the isoelectric point in the form

$$(pH_s)_D = pH_b + \log [Cl]_s - [Cl]_b . \qquad (4a)$$

From this it follows that pH_s is a function of the salt concentration in the bulk phase. The actual degree of dissociation in the surface phase depends primarily on the pH_s, and not on pH_b: hence it follows that, at the same pH_b, a protein will be dissociated (as an acid or base) to different degrees when different amounts of salts are present. In other words, the titration curves, that is, curves of acid or alkali combined with the protein plotted against pH_b, will be different

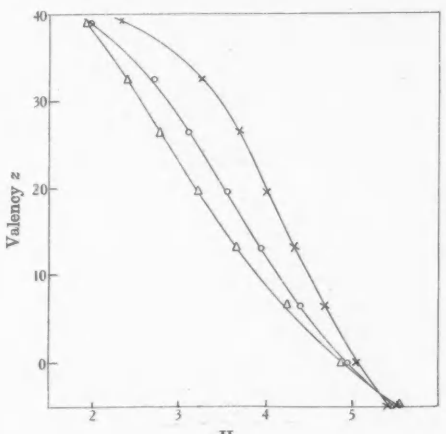


Fig. 2.

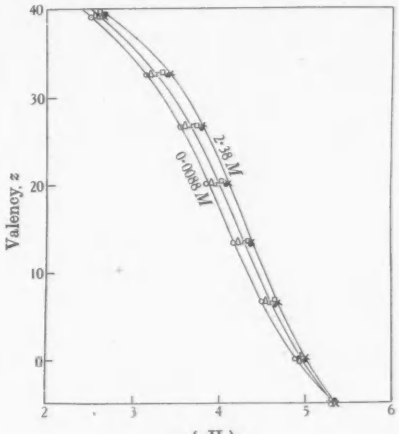


Fig. 3.

Fig. 2. Titration curves of ovalbumin in 2·38, 0·067 and 0·0088 *M* KCl solution. Valency z plotted against pH_b. ×—×—× 2·38 *M*, △—△—△ 0·0088 *M*.

Fig. 3. Titration curves of ovalbumin in various dilutions of KCl. Valency z plotted against pH_s. The 3 curves are for 2·38, 0·067 and 0·0088 *M* KCl. ×—×—× 2·38 *M*, •—•—• 0·67 *M*, □—□—□ 0·133 *M*, ×—×—× 0·067 *M*, △—△—△ 0·033 *M*, o—o—o 0·0088 *M*.

for different salt concentrations. If, however, we were to plot the amount of combined acid or alkali against pH_s, we should eliminate the effect of variation in pH_s with salt concentration. Of course, pH_s cannot be determined directly, but it can be calculated by either of the methods given above.

Consider ovalbumin: Cannan [1938] has published titration curves for this protein in nine different concentrations of KCl. Fig. 2 shows his curves for 0·0088, 0·067 and 2·38 *M* KCl. The pH_b at which a given amount of acid is combined with the protein, i.e. at which the protein has a given valency *z*, varies by up to 0·9 *p*H unit between 0·0088 and 2·38 *M* KCl; as would be predicted from equation (4*a*), the more dilute the bulk phase, the more acid is the pH_b at which a given valency is achieved.

Fig. 3 shows the same data, plotted against pH_s as calculated from equations (3) and (4). The greater part of the variation with salt concentration is eliminated, and instead of the family of curves of varying form of Fig. 2, we get a

series of curves which are substantially the one curve displaced along the $(pH_s)_D$ axis. The method by which $(pH_s)_D$ was calculated was as follows. (1) Approximate values for the surface concentration C_s of the ion of opposite sign to the protein were calculated for various dilutions of KCl. This involved calculation of (*a*) δ, the thickness of the double layer, (*b*) V, the volume of the double layer, and (*c*) the approximate value of C_s as the valency z multiplied by a constant typical of a given salt concentration. Table 1 shows this calculation. The next step (2) was to calculate the approximate values of C_s for a given z but different KCl dilutions from the values in Table 1, then correct this value of C_s by the method of successive approximations. Then (3) $(pH_s)_D$ was finally calculated from equation (3) or (4). A typical example of such calculations is shown in Table 2. Values of $\Delta pH = (pH_s)_D - pH_b$ are also given and will be observed to increase with increasing dilution of KCl. The individual values of $(pH_s)_D$ are subject to an error not greater than $\pm 0{\cdot}05$ *p*H unit, apart from any systematic error.

Table 1. *Calculation of the approximate concentration* C_s *in the surface phase of the ion of opposite sign of charge to that of ovalbumin*

C_s is given in the last column as a function of the valency z of ovalbumin. The radius r of ovalbumin is taken as $2{\cdot}75 \times 10^{-7}$ cm. $\delta = 3{\cdot}1 \times 10^{-8} \div \sqrt{C_b}$.

C_b	$\delta \times 10^7$	$(r+\delta) \times 10^7$	$(r+\delta)^3 \times 10^{21}$	$V \times 10^{20}$	$C_s \times 10^{-2}$ (1st approximation)
2·38	0·21	2·96	25·9	2·18	7·6z
1·19	0·28	3·03	28	3·06	5·4z
0·67	0·38	3·13	30·6	4·15	3·99z
0·27	0·60	3·35	37·5	7·05	2·35z
0·133	0·85	3·60	46·5	10·8	1·53z
0·067	1·20	3·95	61·5	17·1	9·7z
0·033	1·70	4·45	88	28·2	5·85z
0·017	2·38	5·13	134	47·5	3·49z
0·0088	3·3	6·05	222	84·5	1·95z

Table 2. *Calculation of* $(pH_s)_D$ *from the data of Table 1, and equations* (7), (8) *and* (4a)

Case of $z = 26{\cdot}3$.

C_b	C_s 1st approximation	C_s corrected	pH_b	$(pH_s)_D = pH_b - \log C_b + \log C_s$	$\Delta pH = (pH_s)_D - pH_b$
2·38	2·0	3·58	3·70	3·88	0·18
1·19	1·42	2·1	3·55	3·81	0·26
0·67	1·048	1·38	3·5	3·81	0·31
0·27	0·62	0·72	3·35	3·78	0·43
0·133	0·402	0·44	3·22	3·74	0·52
0·067	0·255	0·27	3·10	3·71	0·61
0·033	0·154	0·161	2·88	3·61	0·73
0·017	0·092	0·095	2·80	3·55	0·75
0·0088	0·051	0·053	2·75	3·53	0·78

The fact that, when z is plotted against $(pH_s)_D$ we get not one but several curves, shows that variation in ionic strength at constant pH_b has other effects in addition to affecting pH_s. There are probably many factors involved. The liquid junction potential involved in the measurement of pH_b varies slightly with ionic strength. The dissociation constants even of monocarboxylic acids vary slightly with change in ionic strength. In addition, it has been assumed in the above calculations that the surface area of ovalbumin molecules is in-

dependent of ionic strength: this may well not be true. Also the possibility that ions may enter the interior of the protein molecule has been neglected.

Hartley & Roe [1940] suggest that curves of z plotted against $p\mathrm{H}_s$, rather than $p\mathrm{H}_b$, should be used in determining the nature of the ionizing groups of proteins. It is certainly true that Fig. 3 presents a picture much more susceptible to analysis than is Fig. 2. If, as Cannan suggests, the titration curve of ovalbumin between *p*H 2 and *p*H 7 is that of a polycarboxylic acid, the apparent *p*K of this acid, from Fig. 3, lies between 4·15 and 4·4.

To carry out the suggestion of Hartley & Roe involves calculating $p\mathrm{H}_s$ either from the electrokinetic mobility or from the Gibbs-Donnan equilibrium, using the data of the titration curve itself, as has been done above for ovalbumin. Systematic studies of variation in mobility with wide variation in *p*H and ionic strength have not yet been made, so that the method of Hartley & Roe is not always available. On the other hand, calculation from the Gibbs-Donnan equilibrium involves knowledge of the shape and surface area of the protein concerned—this again is seldom available. A possible solution of this difficulty is to obtain values of the electrokinetic mobility at several *p*H, and from these to calculate the charge density. Then, since the titration curve gives the valency z, i.e. total charge per molecule, multiplying the charge density by z will give the surface area, which may then be used for other *p*H values. This suggestion, however, needs very careful experimental checking.

(3) *The 'protein error' in p*H *determinations with indicators*

Many workers, e.g. Sörensen [1909], have studied the effect of proteins on colorimetric *p*H determinations. As a general rule, where the indicator and protein have opposite charges, the protein error may be large. Where the indicator and protein have the same sign of charge, the protein error is usually small.

Danielli [1937] pointed out that much of the error in *p*H determinations with indicators is due to adsorption of indicators at surfaces whose *p*H is not that of the bulk phase. The indicator, if adsorbed, tends to give the *p*H of the surface phase, not of the bulk phase. Hartley [1934] has made a wide study of the indicator error with solutions containing paraffin chain acids and bases, finding that with solutions containing micelles of basic substances (whose surfaces are more alkaline than the bulk phase) the *p*H given by the indicator is displaced from the bulk value towards the alkaline side, whereas with micelles of acidic substances the displacement is in the reverse direction. A most striking effect is that where warming of the solution causes dissolution of the micelles; as the dissolution becomes complete, the indicator colour changes sharply to that characteristic of the bulk *p*H. This change may be as large as two *p*H units.

A large part of the protein error with indicators is probably due to this effect. Most of the evidence in the literature is fragmentary, but it is true of these scattered observations that with indicators which are anions, the indicator error is large and positive on the acid side of the isoelectric point of a protein and small on the alkaline side. With indicators which are cations the relationships are reversed. This is the type of variation to be expected if the indicator error is mainly due to adsorption of indicator at the protein surface: if a protein is positively charged it will tend to adsorb large organic anions but not cations, and since the *p*H at the protein surface is greater than in bulk, the 'protein error' is positive. These relationships are reversed at a negatively charged surface. St Johnston & Peard [1926] have made a study of the sulphonethalein indicators

with gelatin: their results are in agreement with the above theoretical conclusions, but, as the colour comparison was made by eye, their individual results are in error by 0·05–0·1 *p*H unit, so that the points are scattered rather widely.

Fig. 4 shows some more accurate determinations on 1 % ovalbumin solutions, made with a photoelectric colorimeter, which allows determinations to be made to ±0·02 *p*H unit on standard buffer solutions. The bulk *p*H values were measured with a glass electrode. Three indicators were used: bromocresol green and chlorophenol red being anionic, and neutral red cationic. Fig. 4 shows that with the anionic bromocresol green the protein error is positive on the acid side of the isoelectric point and almost zero on the alkaline side. With chlorophenol red the pH_b values all lay on the alkaline side and the protein error was small. But with neutral red, with which again the pH_b values were all on the alkaline side, the protein error was large and negative. Qualitatively these results are in agreement with theoretical predictions.

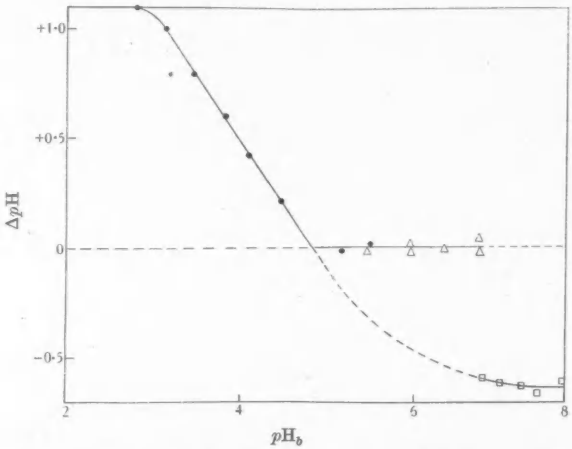


Fig. 4. The protein error, Δ*p*H, for ovalbumin solutions in 0·01*M* NaCl, plotted against pH_b. • Bromocresol green; △ chlorophenol red; □ neutral red.

The ovalbumin was four times recrystallized, dialysed salt-free and diluted to a 4 % solution. NaCl was added to bring the salt concentration to 0·01*N*. 5 ml. samples of the solution were titrated with 0·01*N* NaOH or HCl in 0·01*N* NaCl to suitable pH_b values, with a standard amount of indicator present, and then diluted to 20 ml. with 0·01*N* NaCl: the final solution contained 1 % ovalbumin. The *p*H values of these solutions were measured first with a photoelectric colorimeter, then with the glass electrode, both determinations being made on the same solution, the difference between the two values being the protein error. The colorimeter was calibrated by using solutions of measured *p*H containing 0·01*N* NaCl and 0·0002*M* acetate buffers and the same standard amount of indicator.

Discussion

The previous examination of the results for ovalbumin shows that the *p*H at the surface of ovalbumin molecules may be quite different from that in bulk solution and that it is affected by salt concentration as well as by bulk *p*H. In

the case of colloidal enzymes, as was suggested to me by Prof. J.C. Drummond in 1935, this may be a matter of considerable significance. In 0·01 N solutions, the thickness of the ionic double layer is about 30 Å.: this is considerably greater than the diameter of many substrate molecules, so that in such a solution the enzyme-substrate interaction must occur within the double layer, and the significant pH determining the rate of reaction is probably the surface pH. Varying the ionic strength of the medium at constant $p\mathrm{H}_b$ must vary $p\mathrm{H}_s$, and it seems probable that the greater part of the effect of neutral salts on enzymes is due to this variation in $p\mathrm{H}_s$: Whether this is actually so must await more systematic studies than are yet available. It is, of course, not practicable in general practice to measure $p\mathrm{H}_s$ in most cases. But if the ionic strength of the substrate is kept constant when the pH is changed a standard set of conditions will result, so that one observer can compare his results exactly with those of another; e.g. if two sets of results are obtained with an ionic strength of 0·1, they may be compared exactly, for the same colloid in both sets of experiments will have the same $p\mathrm{H}_s$ at a given $p\mathrm{H}_b$, even though the $p\mathrm{H}_s$ values cannot be measured. The simplest procedure is probably to carry out all experiments in say 0·5 or 0·1 N NaCl, and not to allow the molar concentration of buffer substances to rise above 1 % of the NaCl; under these conditions the ionic strength will be practically constant. Polyvalent ions are to be avoided as far as possible.

Both methods of calculating $p\mathrm{H}_s$ determine the average pH in the surface phase, assuming that the charges are evenly distributed over the colloid surface and it is assumed that $p\mathrm{H}_s$ is the same throughout the surface phase. It is quite possible that this is not true and that there is a considerable local variation in $p\mathrm{H}_s$. Neither method of calculation can detect such local variations. It is possible, however, that the results with indicators are partly due to local variations. The protein error ΔpH of Fig. 4 is roughly 1·4 ($p\mathrm{H}_s - p\mathrm{H}_b$) of Fig. 1, for indicators of opposite charge to the protein; i.e. although qualitatively the protein error follows the difference between the surface and bulk pH values, quantitatively it is 40 % larger than can be due to the calculated values of $p\mathrm{H}_s$, even if the whole of the indicator were adsorbed. This factor of 1·4 may be due to the ionizing groups being concentrated in patches on the protein surface. But there are many other possible explanations and no conclusion can be reached until the distribution of other ions is studied.

The views advanced here are not intended to constitute a complete theory of the protein error with indicators. Many other factors, such as the different valencies of the two forms of an indicator, and molecular interactions such as have been recently studied by Schulman & Rideal [1937], must also be involved.

Summary

1. $p\mathrm{H}_s$, the pH at the surface of a protein molecule, is different from that in the surrounding bulk phase. $p\mathrm{H}_s$ may be calculated either from the Gibbs-Donnan equilibrium, or from the electrokinetic mobility. For ovalbumin the two methods give results in reasonable agreement.

2. At constant bulk pH, $p\mathrm{H}_s$ varies with ionic strength, and consequently the acid- and base-binding powers of a protein vary with ionic strength. It is shown that this accounts for the major part of the influence of ionic strength on the titration curve of ovalbumin, between pH 2 and pH 5·5.

3. Indicators of opposite charge to a protein molecule tend to be adsorbed and give values of $p\mathrm{H}_s$ rather than bulk pH values. Results are given for ovalbumin solutions with bromocresol green, chlorophenol red and neutral red. It is

not clear whether the whole of the protein error is due to the difference between $p\mathrm{H}_s$ and the bulk pH.

4. It is suggested that $p\mathrm{H}_s$, and not $p\mathrm{H}_b$, is the significant pH in enzyme activity, and that, to facilitate comparison of results, enzyme activities should be determined in solutions of constant ionic strength.

I am deeply indebted to Dr G. S. Adair, Dr A. Neuberger and Dr J. H. Schulman for reading the manuscript and for a number of valuable suggestions.

REFERENCES

Abramson (1934). Electrokinetic Phenomena. New York.
Adair & Adair (1940). *Trans. Faraday Soc.* **36**, 26.
Cannan (1938). *Cold Spring Harbour Symposia*, **6**, 1.
Danielli (1937). *Proc. roy. Soc.* B, **122**, 155.
Hartley (1934). *Trans. Faraday Soc.* **30**, 444.
—— & Roe (1940). *Trans. Faraday Soc.* **36**, 105.
Harvey & Dánielli (1938). *Biol. Rev.* **13**, 319.
Kekwick & Cannan (1936). *Biochem. J.* **30**, 227.
Linderstrøm-Lang (1924). *C.r. Carlsbad*, **15** (7).
—— & Lund (1926). *C.r. Carlsbad*, **16** (5).
Schulman & Rideal (1937). *Proc. roy. Soc.* B, **122**, 29, 46.
St Johnston & Peard (1926). *Biochem. J.* **20**, 817.
Sörensen (1909). *Biochem. Z.* **21**, 255.

49. THE METABOLISM OF RAT LIVER DURING CARCINOGENESIS BY BUTTER YELLOW

By JOHN WASHINGTON ORR and LEONARD HUBERT STICKLAND

From the Department of Experimental Pathology and Cancer Research, University of Leeds

(*Received 7 March 1941*)

The production of liver tumours in rats by the inclusion of azo-dyes in their diet was first demonstrated by Yoshida [1932; Sasaki & Yoshida, 1935]. In this work the dye used was *o*-aminoazotoluene (2:1:1:4:3-tolueneazoaminotoluene). Later butter yellow (dimethyl yellow; *p*-dimethylaminoazobenzene) was found to produce the same result more quickly and with a higher yield of tumours [Kinosita, 1937]. The whole subject has been thoroughly reviewed by Kinosita [1937].

These tumours were shown [Nakatani *et al.* 1938] to possess the type of metabolism which is, according to the majority of observers, characteristic of tumours in general, viz. a high rate of anaerobic glycolysis and a considerable rate of aerobic glycolysis. Normal rat liver, on the other hand, according to Warburg *et al.* [1924] shows a low anaerobic glycolysis ($Q_{CO_2}^{N_2} \sim 4$) and negligible aerobic glycolysis ($Q_{CO_2}^{O_2} \sim 1$). The Japanese workers further found that during the period preceding the appearance of a tumour the liver showed a progressively increasing anaerobic glycolysis, from the normal value of $Q_{CO_2}^{N_2} \sim 4$ up to $Q_{CO}^{N_2} \sim 12$. The aerobic glycolysis meanwhile remained at its normal low level. When tumours appeared, they showed the type of metabolism usually associated with tumours, while the remainder of a tumour-bearing liver retained its previous metabolism (high anaerobic, low aerobic glycolysis).

In our first experiments on this subject we were unable to obtain consistently the low values of $Q_{CO_2}^{N_2}$ in normal liver which are quoted by Warburg *et al.* [1924] and Nakatani *et al.* [1938]. Our values ranged indiscriminately between 3 and 15. On consulting the literature, we found that we and the Japanese workers had overlooked the work of Rosenthal [Rosenthal & Lasnitzki, 1928; Rosenthal, 1929], which fundamentally changes the nature of the problem. Rosenthal's results would seem to suggest that (1) liver is not able to glycolyse glucose added to the external medium, but only the glycogen contained in the cells, (2) the rate of this autoglycolysis depends on the state of nutrition of the rat, i.e. presumably on the concentration of glycogen in the liver. In starved animals $Q_{CO_2}^{N_2}$ may be as low as 1, in well-fed ones as high as 16.[1]

[1] Rosenthal found that liver slices placed directly in N_2 showed only a small glycolysis, the high values in well-fed rats being shown by slices removed to N_2 after preliminary incubation in O_2. 30 min. incubation in O_2 at either 20° or 37° sufficed to produce the maximum value of $Q_{CO_2}^{N_2}$. We have failed to observe this phenomenon, and our results under conditions of 'direct' anaerobiosis obviously correspond with his after a preliminary aerobiosis. Perhaps there was a greater delay in our case (up to 20 min. from the killing of the rat to the establishment of anaerobiosis), and our slices had in effect been incubated in air at 20° for a sufficient period to raise $Q_{CO_2}^{N_2}$ to its maximum value.

These considerations make the interpretation of the results of Nakatani *et al.* [1938] very difficult, and we decided to study the following points:

(1) The highest value of $Q_{CO_2}^{N_2}$ recorded by Nakatani *et al.* for liver slices before the appearance of a tumour lies well within our normal range. Is there any evidence of an increase of anaerobic glycolysis of the liver during butter yellow feeding?

(2) Normal liver does not glucolyse. Does butter yellow feeding cause the liver to acquire the power of glucolysis at any stage before the appearance of a tumour?

(3) The glycolytic mechanism of all other tumours so far investigated acts only on monosaccharides. Is this also true of the tumours produced in liver?

(4) There are two main types of carcinomata produced in liver by the action of butter yellow, one derived from the liver parenchyma (hepatomata), and the other from bile-duct epithelium (cholangiomata). In normal liver the liver cells predominate to such an extent that the values for the metabolism of the tissue may be taken to represent the metabolism of the liver cells only. The metabolism of normal bile-duct epithelium is therefore unknown. Do liver cells, when they form tumours, show an abrupt change in metabolism from glycogen glycolysis to glucolysis?

This paper provides an answer to the first three of these questions. On account of the breaking off of our experiments in September 1939, the answer to the fourth remains for the present only tentative.[1]

Experimental

Administration of butter yellow to rats. White stock rats, obtained from a dealer, were used. At the start of the experiment they were almost, but not quite, full-grown. The first group were fed on a mixture of wheat, maize and oats, with supplements of green vegetables and water *ad lib.*; in a later group the cereal food was unpolished Indian rice, as there is evidence [Ando, 1938] that the changes develop more rapidly in rice-fed than in wheat-fed animals. There was no significant difference in the yield or rate of appearance of tumours, or in the findings now reported, as between these two experiments.

The dye used was 'dimethyl yellow (Analar)' and was obtained from British Drug Houses. It was dissolved in warm olive oil in a concentration of 3 %; on cooling, a very small amount crystallized out, and the saturated solution was added to, and intimately mixed with, the cereal part of the animals' food in the proportion of 20 or 30 ml./kg.

It is important to point out that the changes in the livers of our animals progressed at a much slower rate than has been described by the Japanese workers. The detailed histology has been fully described elsewhere [Orr, 1940]. Briefly, the earliest changes in the liver parenchyma are of a degenerative type. After a month, microscopical examination shows proliferation of connective tissue in the neighbourhood of the portal tracts. Later there is added to this regenerative proliferation of both liver tissue and bile ducts, in varying proportions. After about 3 months, as a result of all these processes, the liver has a

[1] An attempt since then to amplify our observations has been unsuccessful as a result of the failure of the rats to develop tumours after up to 15 months of butter yellow feeding. The principal difference has been that these rats have been fed on ordinary white bread with greenstuff supplements. In the original experiments the main diet was unpolished rice, or a mixture of wheat, maize and oats. We have not yet found it possible, under present circumstances, to investigate this problem.

granular appearance similar to that of ordinary cirrhosis, and histologically shows marked replacement of the normal lobular architecture by regeneration nodules of parenchyma and trabeculae of granulation tissue; this stage may conveniently be termed 'nodular hyperplasia'. Carcinomata were first found in the 5th month of treatment, the earliest ones being derived from bile duct epithelium (the so-called cholangiomata); tumours of liver cells proper (hepatomata) appeared rather later, but frequently both types of carcinoma were found in the same liver. It was therefore important that the actual tumour whose metabolism was measured should also be subjected to histological examination; this was done in all instances.

Measurement of metabolism. The manometric methods employed were those described by Berenblum *et al.* [1936], but tissue slices were always used. It must be noted that this method of measuring the aerobic glycolysis, unlike that of Warburg [1924], gives only the glucolysis; both anaerobic methods of course measure the whole lactic acid production from whatever carbohydrate substrates may be present. For the determination of the rate of aerobic glycolysis in normal liver, therefore, the chemical method of lactic acid estimation has been used [Friedemann *et al.* 1927]. Manometric and chemical determinations in the same (anaerobic) experiment showed satisfactory agreement, the manometric figures being usually some 10 % higher than the chemical (cf. Table 7).

Estimation of glycogen concentration in liver. The usual method was used, the details being according to Hynd & Rotter [1930].

I. $Q_{CO_2}^{N_2}$ *of normal liver tissue*

As a result of our earliest experiments and consideration of Rosenthal's work, we decided that the estimation of liver glycolysis, without simultaneous

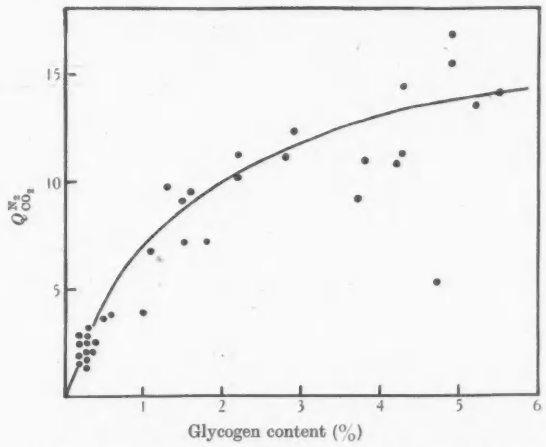


Fig. 1. Rate of glycolysis and glycogen content of normal livers.

determination of the glycogen content of the tissue, yielded results the interpretation of which was necessarily obscure. A number of rats with different liver glycogen concentrations was therefore used to establish the relationship between rate of glycolysis and glycogen concentration. Attempts were made to

produce different glycogen contents by injection of varying amounts of glucose solution into starved rats at appropriate times before killing the animals, but such methods were found to be unreliable. Eventually sufficient liver glycogen values in the range from 1 to 5 % were obtained by random sampling from fed rats, and values from 0·1 to 0·5 % from rats starved for periods up to 48 hr.

The results are shown in Fig. 1. The points are rather scattered, but there is obviously an association between rate of glycolysis and glycogen concentration. It is seen that normal rat liver frequently shows a $Q_{CO_2}^{N_2}$ value of 15 or more, while the highest figure recorded by Nakatani *et al.* [1938] for the liver of rats fed on butter yellow was 11.

It is evident that a comparison of the average values of $Q_{CO_2}^{N_2}$ for a large number of rats of different liver glycogen levels is not a good criterion for determining whether butter yellow feeding induces any change in the rate of glycolysis. In practice, a fairly satisfactory comparison can be made by considering only those cases in which the liver glycogen was 1·5 % or greater. It can be seen from Fig. 1 that from 1·5 to 5 % glycogen the rate of glycolysis increases relatively slowly. Such data for normal livers are shown in Table 1.

Table 1. *Normal liver slices.* $Q_{CO_2}^{N_2}$ *and glycogen concentration*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
16·8	4·9	11·3	2·2	9·4	1·6
15·4	4·9	11·3	4·3	9·1	3·7
14·4	4·3	11·0	3·8	9·1	1·5
14·1	5·5	11·1	2·8	7·3	1·8
13·5	5·2	10·8	4·2	7·1	1·5
12·3	2·9	10·2	2·2	5·2	4·7
			Average	11·1	3·4

II. $Q_{CO_2}^{N_2}$ *of liver tissue from rats receiving butter yellow*

Nakatani *et al.* [1938] related the increase in rate of glycolysis of liver slices solely with the duration of butter yellow feeding. We find that the response to the butter yellow is very irregular; for instance, after some 3 months of feeding, some livers are macroscopically almost normal while others show advanced cirrhosis. In Table 2 we have therefore classified the livers roughly on the basis of the histological results (*v. supra*), under the following headings:

(*a*) Normal.

(*b*) Degenerative changes only.

(*c*) Periportal connective tissue proliferation without macroscopical granularity.

(*d*) Nodular hyperplasia with loss of architecture.

There is clearly no evidence here of a progressive increase in the rate of glycolysis parallel with the changes produced by the feeding of butter yellow.

In Table 3 we have analysed the data for the 20 livers of Table 2 (*c* and *d*), grouping them according to their glycogen contents, and comparing their glycolytic rates with those of a similar series of 18 livers from normal rats. It will be seen that while in both series there is the expected rise in glycolysis as glycogen content increases, in no case is the glycolysis of the butter yellow livers significantly higher than that of the corresponding normal groups. All these differences fall far below the conventional level of significance ($D/\text{S.E.} = 2$).

Table 2. $Q_{CO_2}^{N_2}$ *of livers from rats fed with butter yellow*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
(*a*) Livers which after various periods of butter-yellow feeding were histologically normal:					
14·1	5·5	11·1	2·8	9·1	1·5
13·5	5·2	9·8	3·8		
			Average	11·5	3·7
(*b*) Livers showing degenerative changes:					
11·6	3·0	11·0	3·6	6·4	5·0
			Average	9·7	3·9
(*c*) Livers showing periportal changes:					
14·4	4·0	8·7	2·6	6·7	1·9
14·3	4·1	7·9	3·7	6·4	3·7
13·2	5·5	7·7	3·2	4·5	2·8
12·9	3·0	7·5	2·0	3·7	2·1
11·8	3·1				
			Average	9·2	3·3
(*d*) Livers showing nodular hyperplasia:					
16·6	2·6	13·2	5·1	12·8	4·4
14·2	5·3	13·0	4·2	11·0	4·7
13·5	1·7				
			Average	13·5	4·0

Table 3. *Comparison of precancerous livers of butter yellow-fed rats with normal livers of similar glycogen content*

Range of glycogen %	Average $Q_{CO_2}^{N_2}$ ±standard error		
	Normal	Butter yellow	*D*/S.E.
1·5–2·5	9·1±0·5	7·8±2·1	−0·6
2·5–3·5	11·7±0·6	10·4±1·7	−0·7
3·5–4·5	11·3±0·9	11·5±1·4	+0·1
4·5–5·5	13·4±2·0	12·9±0·7	−0·2

III. *Glycolysis in regenerating liver*

Regeneration of surviving parenchyma plays an important part in the alteration of liver structure during cirrhosis, so for comparison we have made some measurements on regenerating livers. After removal of about one-half of the organ, a rat's liver regains its normal weight in about 8 days, and the maximal rate of growth, as estimated by the number of cells in mitotic division, occurs at about the 4th day. A number of livers were therefore studied on the 3rd, 4th or 5th day after the removal of one-half of the liver. The results are given in Table 4, and it is plain the $Q_{CO_2}^{N_2}$ of regenerating liver does not appreciably differ from that of normal liver.

Table 4. *Glycolysis of regenerating livers*

$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %	$Q_{CO_2}^{N_2}$	Glycogen %
16·7	4·96	14·9	6·20	13·4	3·32
6·7	2·40	15·1	3·18	14·4	3·95
14·7	4·13	12·3	4·30		
			Average	13·5	3·55

IV. *Absence of glucolysis in liver tissue*

In our series of measurements on normal liver there was apparent in general a slightly greater glycolytic rate in the presence of glucose than in its absence, and this difference was not appreciably altered by butter yellow feeding (Table 5). At the same time, we do not believe that this finding is in disagreement with the observations of Rosenthal & Lasnitzki [1928] and Dickens & Greville [1932] that normal liver does not glucolyse, because the differences are small, not invariable, and can be interpreted in more than one way (*v. infra*). The results of Rosenthal [1929] are in agreement with ours in showing a slight increase of $Q^{N_2}_{CO_2}$ in presence of glucose, though he makes no comment on this fact. A curious point is that rat liver readily attacks fructose, and that fructolysis is slight in starved animals and considerable in fed ones [Rosenthal, 1930; Dickens & Greville, 1932].

Table 5. *Glucolysis in liver tissue*

$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$		$Q^{N_2}_{CO_2}$	
Glucose absent	Glucose present	Glucose absent	Glucose present	Glucose absent	Glucose present
		(*a*) Normal and regenerating:			
2·1	2·8	2·2	2·8	5·8	7·4
0·5	1·3	1·2	1·7	3·7	5·0
9·3	8·0	8·2	7·1	12·3	12·0
4·8	5·5	6·7	7·0	15·1	17·6
16·7	17·6	5·8	7·1	2·7	3·5
3·0	3·7	1·8	2·4	3·1	3·2
2·3	3·1	0·8	2·3	14·1	12·7
13·5	16·2	9·7	8·4	11·1	11·1
9·1	6·9	9·3	8·0	8·2	7·1
			Average	6·8	7·1
		(*b*) Degenerative changes:			
11·0	12·7	11·6	11·3	6·4	9·4
			Average	9·7	11·1
		(*c*) Periportal changes:			
14·3	14·5	7·7	9·4	7·9	8·7
14·4	14·2	6·7	4·8	8·7	8·5
6·4	5·2	3·7	7·7	13·2	17·0
4·5	5·2	7·5	14·8	12·9	13·1
11·8	14·3	1·8	4·5	3·0	3·4
			Average	8·5	9·7
		(*d*) Nodular hyperplasia:			
13·0	16·9	12·8	16·6	13·2	14·2
11·0	13·2	13·5	15·2	1·9	4·6
2·0	4·4	14·2	15·4	3·1	7·3
16·6	12·2	4·5	7·7		
			Average	9·6	11·6

It is clear from these figures that there is no striking appearance of glucolysis during the feeding of butter yellow to rats, indeed it would require a very large number of observations to support a claim that there was any significant amount of glucolysis at all.

The same result is found with regenerating livers (Table 6).

Aerobically also liver slices glycolyse only glycogen. A large number of manometric measurements of aerobic glucolysis gave for $Q^{O_2}_{CO_2}$ an average figure

Table 6. *Glucolysis in regenerating livers*

$Q_{CO_2}^{N_2}$	
Glucose absent	Glucose present
16·7	17·6
5·8	7·1
15·1	17·6
4·8	5·5
6·7	7·0
Average 9·8	11·0

of $1{\cdot}1 \pm 1{\cdot}1$, which is not significantly different from zero, while chemical estimation of the total aerobic glycolysis (lactic acid formation) gave the results in Table 7 (the anaerobic results for the same livers are given for comparison, simultaneous chemical and manometric estimations being given side by side to show the degree of agreement).

Table 7. *Glycolysis of normal liver slices*

The paired values in each column are derived from duplicate experiments with material from the same source.

$Q_{CO_2}^{N_2}$ (manometric)		$Q_{CO_2}^{N_2}$ (chemical)		$Q_{CO_2}^{O_2}$ (chemical)	
8·5	9·3	8·7	10·0	1·9	1·8
4·7	4·6	5·6	5·2	1·9	1·7
10·2	10·7	9·4	10·0	2·2	2·4
4·3	4·6	4·3	4·5	1·6	1·5
4·5	4·5	5·3	4·4	1·8	1·9
2·9	3·4	2·4	2·8	1·5	1·3
10·5	10·8	10·4	10·7	1·9	—
8·3	9·5	8·1	9·7	1·9	1·9

V. *The metabolism of the tumours induced by butter yellow*

Owing to the premature end of our experiments, our data on the metabolism of butter yellow tumours are scanty. They are collected in Table 8.

Table 8. *Metabolism of liver tumours*

Type of tumour	$Q_{CO_2}^{N_2}$ Glucose absent	$Q_{CO_2}^{N_2}$ Glucose present	$Q_{CO_2}^{O_2}$ (glucolysis only)
Cholangioma	—	5·5	2·5
,,	—	13·2	5·2
,,	—	13·0	5·4
Hepatoma	—	20·8	8·6
,,	0·0	17·0	6·0
,,	1·9*	8·0*	—
,,	—	11·4	3·2

* This was not a single discrete tumour, but a number of separate nodules. The slices were cut so as to include as much tumour tissue as possible, but they were contaminated with some liver tissue.

Only two results are available which show directly the presence of anaerobic glucolysis in hepatomata. However, in the cases of the three cholangiomata and of the other two hepatomata there is a considerable aerobic glycolysis. As was stated before, our method of measuring aerobic glycolysis manometrically

excludes the lactic acid production from any substrate except the added glucose, so these results all support the view that glucolysis does occur, both in cholangiomata and in hepatomata.

Discussion

From our results we may attempt to answer the four questions mentioned in the introduction (p. 480):

(1) There is no evidence of an increase in the rate of anaerobic glycolysis of liver tissue of rats at any stage of feeding of butter yellow. The increase found by Nakatani *et al.* [1938], unless there is a difference in behaviour between the strains of rats used by them and by us, must have been due to chance. They found an increase of $Q_{CO_2}^{N_2}$ from 4 in normal livers to 12 in cirrhotic livers, while we find that $Q_{CO_2}^{N_2}$ may lie anywhere between 2 and 16 in any rat, according to the glycogen concentration in the liver.

(2) Liver tissue does not acquire an increased power of glucolysis at any stage, prior to actual neoplasm, of butter yellow feeding. The presence of glucose causes an average increase of about 1 in the value of $Q_{CO_2}^{N_2}$, which increase is independent of the absolute value of $Q_{CO_2}^{N_2}$, i.e. is independent of the glycogen content. Among possible explanations of this are (*a*) that liver cells have a slight power of glucolysis, (*b*) that a small proportion of the cells of the liver (e.g. possibly the bile-duct epithelium as distinct from the liver cells) are glucolytic, or (*c*) that the presence of glucose increases the rate of glycogen breakdown.

(3) All the liver tumours we have studied possessed the power of glucolysis. In the two cases in which the point was specially studied, it was found that there was no autoglycolysis, i.e. no glycolytic breakdown of glycogen, and only glucose was attacked. However, histologically the tumour tissue fixed in alcohol was free from glycogen, so there is no possibility of testing whether the tumours would attack glycogen if that substrate were present.

(4) The answer to this important question depends on the results of only four experiments, so we give it only tentatively, and hope to obtain further data in confirmation later. It appears probable, however, that in the transition to malignancy there occurs abruptly a complete change in the type of metabolism of the liver cells, viz. the sole substrate used for glycolysis changes from glycogen to glucose.

Warburg was the first to show a qualitative difference in metabolism between normal and tumour tissues, his results showing that tumour metabolism was characterized by a high rate of glycolysis which was only partially suppressed by oxygen. Since then various normal tissues have been shown to have a similar type of metabolism, e.g. testis (rat), retina (rat) and hyperplastic tonsil (man) [Warburg *et al.* 1924], embryonic lens of rat [Fujita, 1928], kidney medulla in several species [György *et al.* 1928; Dickens & Weil-Malherbe, 1936] and bone marrow of rabbit [Orr & Stickland, 1938], so its specificity to tumours is by no means complete. Recently the criticism has been made [Berenblum *et al.* 1940] that the metabolism of a tumour has in no case been compared with that of the normal tissue from which it is derived, because the normal tissues in question cannot be obtained in sufficient quantity for use with the apparatus so far available. With their new micro-apparatus they have shown that skin epithelium has a metabolism qualitatively the same as that of tumours derived from it, and suggest that the same may be true of all tumours.

Our present results, if confirmed, prove on the contrary that liver cells at any rate do show a sudden change in the type of their metabolism when they form tumours.

SUMMARY

Contrary to the findings of Nakatani *et al.* [1938] we have not observed any change in the glycolytic metabolism of liver tissue in the precancerous phase of butter yellow treatment.

On the other hand, the tumours produced by this agent show a qualitative difference in metabolism from that of normal and cirrhotic liver, the substrate for glycolysis being glycogen in liver tissue and glucose in the tumours derived from it.

The tumours show an aerobic glucolysis equivalent, on the average, to rather more than one-third of the anaerobic glycolysis.

REFERENCES

Ando (1938). *Gann*, **32**, 252.
Berenblum, Chain & Heatley (1940). *Amer. J. Cancer*, **37**, 367.
—— Kendal & Orr (1936). *Biochem. J.* **30**, 709.
Dickens & Greville (1932). *Biochem. J.* **26**, 1546.
—— & Weil-Malherbe (1936). *Biochem. J.* **30**, 659.
Friedemann, Cotonio & Schaffer (1927). *J. biol. Chem.* **73**, 335.
Fujita (1928). *Biochem. Z.* **197**, 175.
György, Keller & Brehme (1928). *Biochem. Z.* **200**, 356.
Hynd & Rotter (1930). *Biochem. J.* **24**, 1390.
Kinosita (1937). *Trans. Soc. Path. jap.* **27**, 665.
Nakatani, Nakano & Ohara (1938). *Gann*, **32**, 240.
Orr (1940). *J. Path. Bact.* **50**, 393.
—— & Stickland (1938). *Biochem. J.* **32**, 567.
Rosenthal (1929). *Biochem. Z.* **207**, 263.
—— (1930). *Biochem. Z.* **227**, 354.
—— & Lasnitzki (1928). *Biochem. Z.* **196**, 340.
Sasaki & Yoshida (1935). *Arch. path. Anat.* **295**, 175.
Warburg (1924). *Biochem. Z.* **152**, 51.
—— Posener & Negelein (1924). *Biochem. Z.* **152**, 309.
Yoshida (1932). *Proc. imp. Acad. Tokyo*, **8**, 464.

50. THE EFFECT OF THIAMINE (VITAMIN B_1) ON FERMENTATION OF YEAST

By H. LASER

From the Molteno Institute, University of Cambridge

(*Received 26 March 1941*)

THIAMINE (vitamin B_1) is known to affect the metabolism and growth of yeast. It was found to stimulate the growth in concentrations as low as 0·01 μg./ml. of culture medium [Williams & Roehm, 1930]. Not every type of yeast, however, is affected in this way, some yeasts responding only very little to thiamine under the usual growth conditions. Similarly some yeasts only are capable of synthesizing thiamine from comparatively simple media [for lit. see Williams, 1941]. Closely related to the effect of thiamine on growth is its effect on yeast fermentation. This is increased by doses of thiamine of the same order of magnitude as those which stimulate growth [Schultz *et al.* 1937, 1, 2]. A quantitative test for determining such minute amounts by the yeast fermentation method has been described by Atkin *et al.* [1939] who found that the addition of 0·01–0·04 μg. of thiamine per 5 mg. yeast (wet weight) suspended in 3 ml. of medium considerably raises the anaerobic CO_2 output during the 2nd hr. of incubation, and that the increase of fermentation caused by doses of 0·01 to 0·02 μg. is proportional to the amount of added thiamine.

In this paper the experiments described by Atkin *et al.* [1939] have been repeated and some more facts have been gained on the effect of thiamine on yeast fermentation.

EXPERIMENTAL

The experiments were made with ordinary Warburg manometers (conical flasks of about 18 ml.) at 20 and 39°. N_2 was freed from traces of O_2 by passing it over heated copper.

For each vessel 1–5 mg. wet weight of ordinary baker's yeast or *Torula utilis* were used, suspended in the medium employed by Atkins *et al.* Their experimental procedure was generally followed except that the volume of fluid per vessel was always kept constant when different amounts of thiamine were tested (*vide* Table 1). The percentage increase in the rate of fermentation caused by thiamine above that of the control to which no thiamine had been added was plotted against concentration of thiamine.

Table 1

Exp. 12, Fig. 4. Period, 60–110 min. Anaerobic fermentation, 3 mg. yeast per vessel, 20°. Thiamine solution, 0·04 μg./ml. Thiamine test solutions: A, 0·02 μg./ml. B, 0·03 μg./ml.

	I	II	III	IV	V
Distilled water	2·0	1·0	1·75	1·0	1·0
Thiamine solution	—	1·0	0·25	1·0 A	1·0 B
Yeast susp.	1·0	1·0	1·0	1·0	1·0
h (mm.)	+41·0	+81·0	+63·75	+74·0	78·75
k_{CO_2}	1·887	1·893	1·903	1·801	1·823
μl. CO_2	+77·5	+153	+121	+133	+143
% increase above I	—	+97·8 %	+56·5 %	+71·9 %	+85·5 %

Results

Anaerobic fermentation of yeast. Fig. 1 shows the percentage increase of anaerobic fermentation at 20° of 5 mg. yeast per vessel for different periods from 0 to 150 min. induced by thiamine in concentrations of 0·01, 0·02 and 0·04 μg. per vessel. The experiment shows:

(1) There is a very marked increase of anaerobic fermentation under the influence of thiamine.

(2) The percentage increase is in definite relation to the amount of thiamine added.

(3) The absolute amount of CO_2 produced under the influence of thiamine increases with time, the relative percentage increase caused by different thiamine concentrations, however, remains more or less the same for each period.

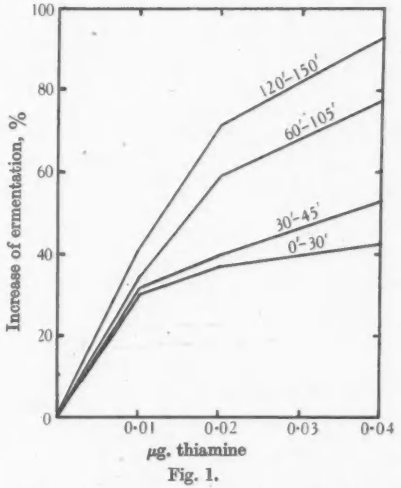


Fig. 1.

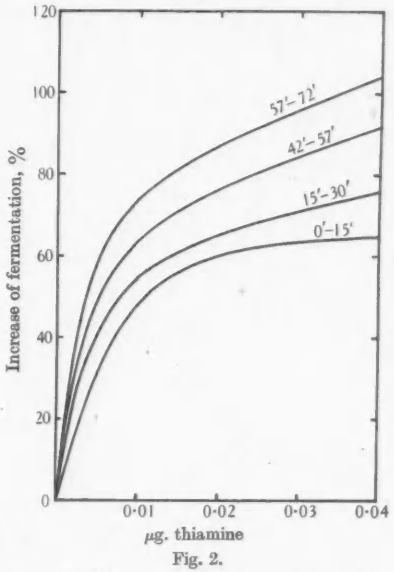


Fig. 2.

Fig. 1. Percentage increase of anaerobic fermentation for different periods of the experiment. 5 mg. yeast (wet wt.) per vessel; 20°.

Fig. 2. Percentage increase of anaerobic fermentation for different periods of the experiment. 2 mg. yeast; 39°.

This last fact probably explains why Atkin *et al.* have used the 2nd hour for their determinations, for it is only in this period that the absolute increase of fermentation caused by a given amount of thiamine is significant enough to reveal comparatively small differences of the fermentation rate caused by different amounts of thiamine.

Fig. 2 shows the percentage increase of anaerobic fermentation by 2 mg. yeast per vessel for different periods from 0 to 72 min. with 0·01, 0·02 and 0·04 μg. thiamine at 39°. On the whole, this experiment closely resembles that of Fig. 1, the same relative increase of fermentation being caused by various amounts of thiamine, and the rate of fermentation increasing with time. The only difference from Fig. 1 is that all the curves lie on a slightly higher level. This represents the

temperature coefficient of the effect, which is evidently low, considering that the temperature difference is about 20°. The higher level of fermentation rate in this experiment does not seem to be caused by the fact that the same amount of thiamine is allowed to act on less yeast per vessel (2 mg. instead of 5 mg.) because the same thiamine concentration acting on half the amount of yeast (1 mg., Fig. 3) but at the same temperature (39°) does not further increase the rate of fermentation. Fig. 2 shows also that the quantitative testing of thiamine need not be restricted to the narrow range between 0·01 and 0·02 μg. where the increase of fermentation is proportional to the amount of added thiamine. If the rates of fermentation are plotted against concentration of thiamine for different consecutive periods of the experiment a similar type of curve is obtained, which allows testing for thiamine over a wider range.

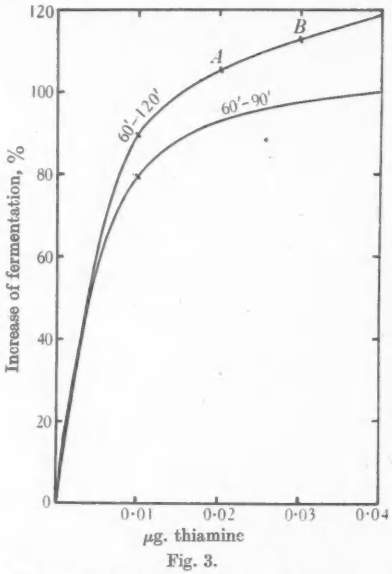


Fig. 3.

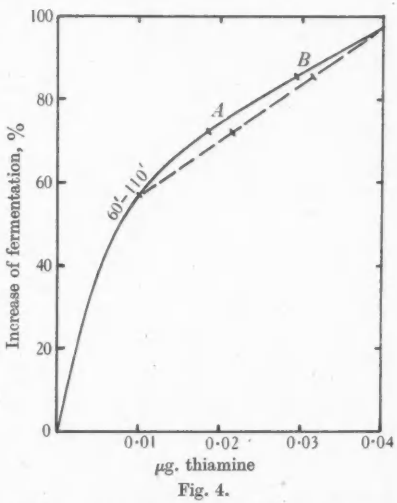


Fig. 4.

Fig. 3. Determination of the thiamine contents of two test solutions, $A = 0{\cdot}02$ μg./1·0 ml., $B = 0{\cdot}03$ μg./1·0 ml., by the percentage increase of anaerobic fermentation. 1 mg. yeast; 39°.

Fig. 4. Same as Fig. 3. 3 mg. yeast; 20°.

Fig. 3 represents the results of such an experiment, in which the strength of two thiamine solutions (*A* and *B*) was determined. Solution *A*, containing 0·02 mg./1·0 ml., and *B*, containing 0·03 μg./1·0 ml., had been given to me without my knowing their thiamine contents. Two consecutive determinations of the fermentation rates were made for the periods 60–90 min. and 60–120 min. with 0·01 and 0·04 μg. thiamine each. The thiamine contents of solutions *A* and *B* were determined by the fermentation rate of the same amounts of yeast for the period 60–120 min. The figure shows that the rates found for *A* and *B* correspond exactly to the amounts of thiamine present, i.e. 0·02 and 0·03 μg.

Fig. 4 (3 mg. yeast; 20°) shows a similar experiment where for the period 60–110 min. the fermentation rate was measured with 0·01 and 0·04 μg. as standard and *A* (0·02 μg.) and *B* (0·03 μg.) as unknown thiamine concentra-

tions. Irrespectively of whether the points marking the fermentation rate with 0·01 and 0·04 μg. were connected by a curve or a straight line, the values obtained for *A* and *B* lie close enough to either of them to allow the respective thiamine contents to be determined sufficiently accurately for all practical purposes.

Aerobic fermentation of yeast. This is quantitatively affected by thiamine in the same way as anaerobic fermentation, but the test can, of course, be made only with yeast varieties which normally have some aerobic fermentation. An example is shown in Fig. 5, where the percentage increase of aerobic fermentation is determined with 0·01, 0·02, 0·03 and 0·04 μg. thiamine, the points marking the fermentation rates with 0·02 and 0·03 μg. lying close to either a curve or a straight line connecting the points which correspond to the fermentation rate with 0·01 and 0·04 μg. of thiamine.

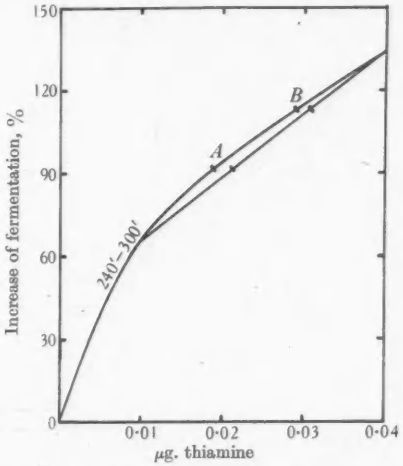


Fig. 5. Determination of the thiamine contents of two test solutions, $A = 0{\cdot}02$ μg./ml., $B = 0{\cdot}03$ μg./ml. by the percentage increase of *aerobic* fermentation. 3 mg. yeast; 20°.

Technically the measurement of aerobic fermentation is simpler and quicker than that of anaerobic fermentation. It requires, however, in addition to the usual manometer (I), another manometer (II) containing KOH in the inner cup for the measurement of respiration (x_{O_2}) during the experimental period. Assuming R.Q. = 1, the respiratory $CO_2 = -x_{O_2}$. If the observed pressure changes and vessel constants are denoted by dashes for their respective vessels, and the aerobic fermentation by x_{FCO_2}, then

$$x_{FCO_2} = k'_{CO_2}\left(h' - \frac{h'' k''_{CO_2}}{k''_{O_2}}\right) + h'' k''_{O_2}.$$

Not every type of yeast responds in the manner described when thiamine is added, and yeast which does respond to the addition of thiamine may differ widely with regard to the time at which the increase of fermentation occurs. Some samples of baker's yeast have been tested at which the characteristic curves have been obtained only after 6–7 hr. incubation with thiamine. In such cases the increase of fermentation by thiamine (0·01–0·04 μg.) during the first hours of the experiments was either absent or more or less uniform at between 10 and

20 %, thus not permitting any distinction between the various amounts of thiamine. After 6–7 hr. the different rates became more and more distinct. In some cases incubation was extended for 24 hr., by which time the differences were very marked. The maximum increase of fermentation caused by the addition of 0·04 μg. seldom exceeded 120–140 % however.

Behaviour of Torula utilis. *Torula utilis* did not respond at all within the period examined (24 hr.) to the applied range of thiamine (0·01–0·04 μg.). Neither aerobic nor anaerobic fermentation is increased above that of the control. On the other hand the rate of anaerobic fermentation of the control without thiamine increases steadily with time by approximately 20–25 % per hr. until it reaches a maximum of approximately 600 % at which level it seems to remain steady. *T. utilis*, without the addition of thiamine, behaves with regard

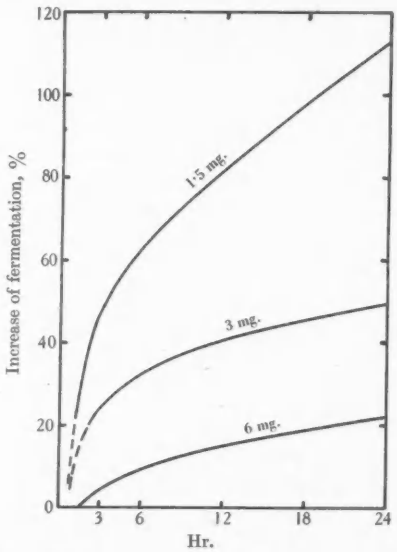


Fig. 6. Percentage increase of anaerobic fermentation caused by the same amount of thiamine (0·04 μg.) acting on different amounts of yeast. 20°.

to its anaerobic fermentation like baker's yeast to which thiamine has been added. This fact seems to justify the conclusion that *T. utilis* is able to synthesize thiamine from the medium and to do this at such a rate that added thiamine (0·01–0·04 μg.) does not further increase the already naturally increasing rate of fermentation. When 0·4 μg. of thiamine was added an increase of fermentation of between 20–30 % was found only after more than 24 hr. incubation. Quantitative tests at this stage of incubation and with these larger doses have, however, not been made.

Torula has only a very small, practically insignificant, aerobic fermentation. Unlike the anaerobic fermentation, this does not increase with time. On the other hand respiration increases at about the same rate as anaerobic fermentation.

While the rate of anaerobic fermentation of *Torula* without thiamine increases, that of baker's yeast as a rule decreases slightly with time. In analogy

to the behaviour of *Torula* which probably synthesizes thiamine it might be concluded that baker's yeast is not only incapable of doing this but is losing some of its own preformed thiamine under the conditions of the experiment.

If this is the case, then different amounts of yeast incubated for long periods with the same amount of thiamine should show differences in the increase of CO_2 production. In the experiment summarized in Fig. 6 a sample of baker's yeast was used which responded only very slowly and which reacted to different amounts of thiamine in the characteristic way only after prolonged incubation. Different amounts (1·5, 3 and 6 mg.) of this yeast were exposed to the same amount of thiamine (0·04 μg.) and the relative increase of CO_2 production determined at different times. Since the volume of fluid was the same for each vessel, the relative amounts of added thiamine per mg. of yeast were in the ratio of 1 : 2 : 4. If preformed thiamine was used up at the same rate in each vessel, then the relative increase with time of CO_2 production caused by 0·04 μg. should be greater with 1·5 mg. than with 6 mg. yeast. This is actually the case, as can be seen from Fig. 6.

The effect of reduced thiamine

Lipmann [1936], who studied the reduction of vitamin B_1 by $Na_2S_2O_4$ and by H_2 in presence of platinum black, remarked on the close similarity of his results to those of Warburg *et al.* [1935] with coenzyme. He suggested that the sulphite-reduced compound corresponds to the naturally reduced component of thiamine in the body and assumed that thiamine in the body is reduced and reoxidized. It was therefore of interest to compare the influence of the oxidized and reduced product on the rate of fermentation of yeast. The following results have been obtained.

(1) The reduction of thiamine by H_2+platinum black yields an irreversibly inactive product, e.g. fermentation in presence of H_2-reduced thiamine, even over prolonged periods, is of exactly the same magnitude as that of the control without thiamine.

(2) When thiamine which has previously been reduced by $Na_2S_2O_4$ (in 0·2 % $NaHCO_3$) is added to a readily reacting type of yeast in N_2 (by tipping it from a side bulb) no difference is found as compared with the effect of the oxidized compound. It must be assumed, therefore, that reduced thiamine is rapidly reoxidized within the yeast cells. The smallest amount of yeast used for these experiments was 1·5 mg. with 0·04 μg. reduced thiamine. If still smaller amounts of yeast could be used, a time lag for the complete reactivation of the reduced thiamine might be expected and a quantitative relation established. The technical difficulties, however, in determining this relation seemed unsurmountable: this is not surprising considering that the analogous reoxidation of reduced coenzyme by flavin comes to a standstill within 5 min.

It may be added that $Na_2S_2O_4$ is itself not without influence on the metabolism of yeast. Even in very small amounts it inhibits respiration and fermentation, the latter to a greater extent. No effect on either respiration or fermentation was obtained when 0·01 mg. or less $Na_2S_2O_4$ was added per 3 ml. of fluid. The reduction of thiamine was therefore carried out in a relatively concentrated solution of thiamine, e.g. 1 ml. 0·2 % $NaHCO_3$ contained 10 μg. thiamine and 2·5 mg. $Na_2S_2O_4$. This was diluted 50 times, and 0·2 ml. was then tipped from the side bulb of the vessel into the yeast suspension under anaerobic conditions. The same amount of equally diluted $NaHCO_3$ solution was tipped into the control vessels without thiamine and with oxidized thiamine.

Conclusions and summary

1. Under the conditions used by Schultz *et al.* thiamine in amounts as low as 0·01 μg. increases the anaerobic fermentation of yeast.

2. The percentage increase of the rate of fermentation by 0·01–0·04 μg. thiamine, plotted against thiamine concentration for several consecutive periods of the experiment, gives a similar type of curve. Thus it is possible to make quantitative tests of unknown concentrations of thiamine within this range with considerable accuracy.

3. Not every type of yeast responds with increased fermentation to the addition of thiamine. Thus baker's yeast was found to react regularly and fairly well, while *Torula utilis* under similar conditions did not respond at all.

4. The time at which responding yeast begins to show an increased fermentation after thiamine has been added varies considerably. Some samples react immediately, some only after several hours of incubation.

5. Once the rise of fermentation has started it increases with time for the same dose of thiamine. Differences in the increase of fermentation caused by different amounts of thiamine acting on the same amount of yeast are therefore more pronounced (and easier to evaluate quantitatively) after some incubation, although the relative increases remain the same.

6. In yeasts which react by an increase of anaerobic fermentation, aerobic fermentation is also increased quantitatively by the same minute amounts of thiamine, the percentage increase being of about the same magnitude as that of anaerobic fermentation.

7. The reaction which causes the increase of fermentation has a low temperature coefficient.

8. Different amounts of a slowly reacting sample of yeast incubated with the same amount of thiamine for 24 hr. show, as time goes on, a relatively greater increase of fermentation with decreasing amounts of yeast.

9. Reduction of thiamine by H_2 in presence of platinum black yields an irreversibly inactive product.

10. The $Na_2S_2O_4$-reduced thiamine, when added to yeast in N_2, affects fermentation in the same way as the oxidized form. It is concluded that the reduction by $Na_2S_2O_4$ is reversible and that thiamine reduced by $Na_2S_2O_4$ is reoxidized within the living yeast cell.

This investigation was suggested by Dr A. C. Thaysen to Prof. D. Keilin. I wish to express my thanks to Prof. Keilin for his interest and advice during the course of the experiments.

References

Atkin, Schultz & Fry (1939). *J. biol. Chem.* **129**, 471.
Lipmann (1936). *Nature, Lond.*, **138**, 1097.
Schultz, Atkin & Fry (1937, 1). *J. Amer. chem. Soc.* **59**, 948.
—— —— —— (1937, 2). *J. Amer. chem. Soc.* **59**, 2457.
Warburg, Christian & Griese (1935). *Biochem. Z.* **282**, 157.
Williams (1941). *Biol. Rev.* **16**, 49.
—— & Roehm (1930). *J. biol. Chem.* **87**, 581.

Note added 26 May 1941. The application of the yeast fermentation method for the quantitative assay of vitamin B_1 in foodstuffs and tissues is limited by the fact that some yeasts are capable of synthesizing vitamin B_1 from certain degradation or split products of the vitamin molecule. These products, however, are not biologically active in animals. The presence of some such products might therefore give a higher fermentation test than warranted by the presence of free thiamine. I am indebted to Dr L. Harris who kindly drew my attention to this point.

51. THE FORMATION OF SUCCINIC ACID IN YEAST

By ARNOŠT KLEINZELLER

From the Department of Biochemistry, University of Sheffield

(*Received 26 March 1941*)

PASTEUR [1860] was the first to observe succinic acid among the substances formed during alcoholic fermentation. In his experiments the yield of succinic acid was 0·5–0·7 % of the fermented sugar. Ehrlich [1909] found that fermenting yeast is capable of converting glutamic acid into succinic acid and advanced the view that all the succinic acid formed during fermentation is derived from glutamic acid; the latter was assumed to arise from the autolysis of dead yeast cells. This view has been widely accepted.

It will be shown in this paper that in addition to Ehrlich's mechanism there is a second mechanism of succinic acid formation in which sugar is the parent substance. Under normal conditions most, if not all, of the succinic acid is derived from this source.

EXPERIMENTAL

Methods. Suspensions of yeast in 1 % KH_2PO_4 or 0·1 *M* phosphate buffer *p*H 7·4, or 0·5 % $NaHCO_3$ were used, and 3 ml. of the suspension containing 2–40 mg. yeast (dry weight) were measured into the main compartment of conical manometer cups. The substrate was measured into the side-arm and the volume brought up to 4 ml. with water unless otherwise stated. The gas space contained air, or expired air (i.e. 5 % CO_2 in air), or, in anaerobic experiments, CO_2. In the anaerobic experiments yellow phosphorus was placed in the centre chamber to remove traces of O_2.

The manometers were shaken at 25°. When equilibrium was established the substrates were added from the side-arm and the cups were shaken until the CO_2 output had almost ended. The yeast suspension was then deproteinized with tungstic acid and the succinate was estimated manometrically [see Krebs, 1937]. The results are expressed in μl. as usual.

In most of the experiments baker's yeast was used, but a few experiments were carried out with brewer's yeast, washed suspensions of pure cultures of *Torulopsis utilis*, or a strain of *Saccharomyces cerevisiae* adapted to the use of galactose (received from Dr Marjory Stephenson, see Stephenson & Yudkin [1936]).

(1) *Effect of media.* Two factors were found to affect the formation of succinate by fermenting yeast: the concentrations of H^+ and of CO_2 and bicarbonate.

Generally no succinate was formed when 1 % KH_2PO_4 was used as a medium, whilst 85–245 μl. succinate were formed from 50 mg. of glucose when 0·5 % $NaHCO_3$ saturated with 5 % CO_2 was used. The importance of the CO_2 pressure is demonstrated by Exps. 1 and 2, Table 1, where two media of approximately equal *p*H but of different CO_2 concentration were compared. No succinate was formed in the absence of glucose.

Table 1. *Succinate formation by baker's yeast in different media*

(1 ml. 5 % glucose; 3 ml. medium.)

No.	mg. yeast (dry wt.)	Medium	μl. succinate found
1 and 2	18·6	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0; 0
		0·1 *M* phosphate buffer *p*H 7·4; air; CO_2 absorbed by NaOH	0; 0
		0·5 % $NaHCO_3$; 5 % CO_2 in air	85; 92
3	19·5	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	37
		0·5 % $NaHCO_3$; 5 % CO_2 in air	152
		0·5 % $NaHCO_3$; deproteinized immediately	15
4	10·0	1 % KH_2PO_4; CO_2	0
	20·0	1 % KH_2PO_4; CO_2	0
	40·0	1 % KH_2PO_4; CO_2	0
	10·0	0·5 % $NaHCO_3$; CO_2	108
	20·0	0·5 % $NaHCO_3$; CO_2	109
	40·0	0·5 % $NaHCO_3$; CO_2	119

(2) *Effect of glucose.* The amount of succinate formed during the fermentation in the presence of $NaHCO_3$ and CO_2 is proportional to the quantity of sugar fermented; it increases linearly with the amount of sugar added and with time. Per 100 mol. of sugar fermented about 1·35–3·96 mol. of succinate are formed. Slightly larger quantities seem to be formed when low concentrations of yeast are used (see Table 2).

The proportion of yeast present to succinate formed, which would be expected to be fairly constant if Ehrlich's hypothesis were correct, shows very wide variations (Table 1, Exp. 4). If a small quantity of yeast is used, the yield of succinate exceeds the total dry weight of yeast (Table 2, Exp. 3). This is conclusive proof of the formation of succinate from added glucose.

Table 2. *Succinate formation from glucose*

Baker's yeast in 3·0 ml. 0·5 % $NaHCO_3$. 5 % CO_2 in air in Exps. 1 and 2; CO_2 in Exp. 3.

No.	mg. yeast (dry wt.)	Glucose added	Duration of exp. (hr.)	μl. succinate formed
1	23·4	0	23	0
		1·0 ml. 5 %	6	134
		1·0 ml. 10 %	8	274
		2·0 ml. 10 %	23	434
2	19·5	0	0	15
		1·0 ml. 5 %	1	19
		1·0 ml. 5 %	3	100
		1·0 ml. 5 %	5	152
3	2·0	1·0 ml. 10 %	90	407 (=2·14 mg.)

(3) *Effect of O_2.* The yield of succinate was found to be the same in aerobic and anaerobic experiments, although fermentation is more rapid in the absence of O_2 (Table 3).

Table 3. *Formation of succinate under aerobic and anaerobic conditions*

20·4 mg. baker's yeast (dry weight) in 3 ml. 0·5 % $NaHCO_3$; 1 ml. 5 % glucose.

No.	Gas mixture	μl. succinate found
1	5 % CO_2 in air	137
	CO_2; phosphorus	148
2	5 % CO_2 in air	175
	CO_2; phosphorus	174

(4) *Succinate formation from various sugars.* The amounts of succinate formed from glucose fructose and sucrose were found to be of the same order. This confirms Pasteur's [1860] results. A strain of *Saccharomyces cerevisiae* adapted to the use of galactose formed the same quantities of succinate from glucose and from galactose.

Table 4. *Formation of succinate from various sugars*

Exp. 1. 23·4 mg. baker's yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; 1 ml. 5 % sugar added.
Exp. 2. 19·3 mg. *S. cerevisiae* adapted to use of galactose, in 3 ml. 0·5 % $NaHCO_3$; 1 ml. 10 % sugar added; 5 % CO_2 in air.

Exp.	Sugar added	μl. succinate found
1	Glucose	164
	Fructose	190
	Saccharose	184
2	0	0
	Glucose	183
	Galactose	151

Table 5. *Effect of various substrates on the formation of succinate in baker's yeast*

(3 ml. yeast suspension, 1 ml. substrate. Medium, 0·5 % $NaHCO_3$; 5 % CO_2 in air, unless otherwise stated. Brackets indicate experiments carried out on the same suspension and therefore directly comparable.)

mg. yeast	Additions	μl. succinate found
23·4	1·0 ml. 0·2 *M* Na acetate	0
	1·0 ml. 0·2 *M* Ba acetate	0
	1·0 ml. 0·2 *M* Na acetate*	0
	1·0 ml. 0·2 *M* Ba acetate*	0
19·5	0	20
	50 mg. glucose	161·5
	1·0 ml. 0·25 *M* glycerol	6·5
	1·0 ml. 0·25 *M* α-glycerophosphate	19
	50 mg. glucose; 0·5 ml. 0·2 *M* pyruvate	157
23·4	50 mg. glucose	149
	50 mg. glucose; 0·5 ml. 0·2 *M* fumarate	138
	50 mg. glucose; 0·5 ml. 0·2 *M* aspartate	184
	1·0 ml. 0·25 *M* fumarate	29
	1·0 ml. 0·25 *M* aspartate	10
19·5	50 mg. glucose	157
	50 mg. glucose; 0·5 ml. 0·2 *M* oxaloacetate	187
	1·0 ml. 0·25 *M* oxaloacetate	24·5
15·5	50 mg. glucose	132
	50 mg. glucose; 0·5 ml. 0·2 *M* *l*-tartrate	138
	1·0 ml. 0·25 *M* *l*-tartrate	0
19·5	50 mg. glucose	134
	50 mg. glucose; 0·5 ml. 0·2 *M* *l*-malate	194
	50 mg. glucose; 0·5 ml. 0·2 *M* α-hydroxyglutarate	183
	1 ml. 0·25 *M* *l*-malate	45
	1 ml. 0·25 *M* α-hydroxyglutarate	21
19·5	50 mg. glucose	160
	1·0 ml. 0·25 *M* α-ketoglutarate	156
19·5	50 mg. glucose	134
	50 mg. glucose; 0·5 ml. 0·2 *M* glutamate	450
	50 mg. glucose; 0·5 ml. 0·2 *M* α-ketoglutarate	174
19·5	50 mg. glucose	141
	50 mg. glucose; 0·5 ml. 0·2 *M* glutamate	493
	50 mg. glucose; 0·5 ml. 0·2 *M* α-hydroxyglutarate	195
15·5	50 mg. glucose	132
	50 mg. glucose; 0·5 ml. 0·2 *M* arginine	131
	50 mg. glucose; 0·5 ml. 0·2 *M* ornithine	138

* 0·1 *M* phosphate buffer; air.

(5) *Effect of other substrates.* The effect of the following substances on the formation of succinate was tested in the presence and in the absence of glucose: acetate, pyruvate, lactate, glycerol, α-glycerophosphate, oxaloacetate, *l*-malate, fumarate, *l*-tartrate, γ-aminobutyrate, *l*-glutamate, α-hydroxyglutarate, α-ketoglutarate, *dl*-ornithine, *l*-arginine. The final concentration of these substances when added alone was 0·0625*M*, when added with glucose 0·025*M* (unless otherwise stated).

In absence of glucose α-ketoglutarate was the only substance to yield succinate [see Neuberg & Ringer, 1915]. In presence of glucose 2- and 3-carbon compounds had no effect on the formation of succinate. Oxaloacetate, *l*-malate and aspartate increased the yield of succinate by about 20 % and α-ketoglutarate and α-hydroxyglutarate about 30 %, whilst fumarate, *l*-tartrate, γ-aminobutyrate, arginine and ornithine had no effect (Table 5).

The succinate formation from *l*(+)-glutamic acid, first shown by Ehrlich [1909], is confirmed. The quantity of succinate formed depends on the composition of the medium; only a small amount is formed when the medium is weakly acid and CO_2 is absorbed by NaOH; considerable quantities appear in 0·5 % $NaHCO_3$ saturated with 5 % CO_2.

No difference was found in the yield of succinate from glutamic acid under aerobic or anaerobic conditions.

Table 6. *Formation of succinate from l(+)-glutamate in baker's yeast*

Glutamate concentration *M*/40 unless otherwise stated.

No.	mg. yeast	Substrates: Glucose (50 mg.)	Substrates: Glutamate	Medium	μl. succinate found
1	23·4	+	–	0·5 % $NaHCO_3$; 5 % CO_2 in air	157
		+	+	,, ,,	500
		–	+*	,, ,,	6
2	18·6	+	–	0·5 % $NaHCO_3$; 5 % CO_2 in air	82
		+	+	,, ,,	402
		+	+	0·1 *M* phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0
3	18·6	+	–	0·5 % $NaHCO_3$; 5 % CO_2 in air	92
		+	+	,, ,,	396
		+	+	0·1 *M* phosphate buffer; air; CO_2 absorbed by NaOH	0
		+	+	1 % KH_2PO_4; air; CO_2 absorbed by NaOH	0
4	20·4	+	–	0·5 % $NaHCO_3$; 5 % CO_2 in air	137
		+	+	,, ,,	305
		+	+	0·5 % $NaHCO_3$; CO_2; phosphorus	330

* 0·0625 *M* glutamate.

Table 7. *Effect of NH_4Cl on the formation of succinate in baker's yeast*

16·1 mg. yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; 50 mg. glucose.

Additions (final conc. 0·025 *M*)	μl. succinate found
0	82
l-Glutamate	364
α-Ketoglutarate	250
α-Hydroxyglutarate	141·5
α-Ketoglutarate; NH_4Cl	78
α-Hydroxyglutarate; NH_4Cl	83
NH_4Cl	92

α-Ketoglutarate and α-hydroxyglutarate have been assumed to be intermediates in the succinate formation from glutamate [Ehrlich, 1909; Neuberg & Ringer, 1915] and it is therefore remarkable that under identical conditions the yields of succinate from these acids were smaller than those from glutamate. This result is not affected by the addition of equimolecular quantities of NH_4Cl (Table 7). Since the permeability of the yeast cell may influence the rate of reaction we do not regard our results as contradictory to the hypothesis of Neuberg & Ringer.

(6) *Effect of growth.* Little or no succinate was found, even in presence of $NaHCO_3$, when the fermentation proceeded in a medium in which growth occurs. Two media were tested, a modified Czapek-Dox medium (2 g. $NaNO_3$, 1·32 g. Na_2HPO_4, $2H_2O$, 0·5 g. $MgSO_4$, $7H_2O$, 0·5 g. KCl, 0·01 g. $FeSO_4$, $7H_2O$ in 1 l. tap water) and the medium of Fink & Krebs [1938] (2·6 g. $(NH_4)_2HPO_4$, 3·3 g. $(NH_4)_2SO_4$, 2 g. K_2SO_4, 1·5 g. $MgSO_4$, $7H_2O$, in 1 l. tap water).

The recovery of succinate added to yeast fermenting in 0·5 % $NaHCO_3$, or 1 % KH_2PO_4, was quantitative, whilst a considerable amount of succinate was taken up by the yeast fermenting in the complex medium (Table 8).

Table 8. *Effect of growth on the formation of succinate in baker's yeast*

20·0 mg. yeast in 3 ml. medium; 5 % CO_2 in air.

		Additions		
No.	Medium	Glucose mg.	Succinate μl.	Succinate found, μl.
1	Czapek-Dox	—	—	0
	"	50	—	39
	0·5 % $NaHCO_3$	50	—	138
2	Fink-Krebs	—	—	0
	"	50	—	0
	0·5 % $NaHCO_3$	50	—	115
3	0·5 % $NaHCO_3$	50	—	247
	"	50	672	943
	Fink-Krebs	50	—	45
	"	50	672	638

(7) *Experiments on brewer's yeast and on* Torulopsis utilis. Experiments with varying quantities of sugar were carried out on fresh brewer's yeast and on a pure culture of *Torulopsis utilis*. As in the case of baker's yeast the yield of succinate was found to be proportional to the quantity of sugar fermented (Table 9).

Table 9. *Succinate formation from glucose in different yeasts*

Exp. 1. 15 mg. brewer's yeast in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; glucose.
Exp. 2. 20·2 mg. *Torulopsis utilis* in 3 ml. 0·5 % $NaHCO_3$; 5 % CO_2 in air; glucose.

No.	mg. glucose added	Duration of exp. (hr.)	μl. succinate found
1	0	0	0
	0	30	10
	50	6½	87
	100	24	167
	200	30	268
2	0	22	0
	25	2½	58
	50	4	107
	100	6½	195
	200	22	236

(8) *Presence of fumarase in yeast.* In view of the fact that fumarase takes part in the synthesis of succinate in some organisms [Krebs & Eggleston, 1940; Smyth, 1940] it was thought of interest to examine the fumarase activity of yeast. Fumarate was estimated according to Krebs *et al.* [1940]. No evidence was found of the presence of fumarase in baker's yeast. Added malate was not converted into fumarate within *p*H 5–8. This explains why malate and fumarate behave differently (Table 5). The conversion of malate into succinate must be regarded as a direct reduction which does not involve the formation of fumarate.

Discussion

(1) *Source of succinate.* The experimental results show that in normal conditions of fermentation sugar is the main parent substance of succinate (Exp. 5, Table 1). The fact that 1 mg. of yeast can form 1·07 mg. succinic acid proves that the succinate cannot be derived from the yeast protoplasm, as assumed by Ehrlich. It must be formed from the added substrate, i.e. glucose.

(2) *Mechanism of succinate formation from glucose.* The formation of succinate depends on the presence of $NaHCO_3$ and of CO_2. A similar relationship between the formation of C_4-dicarboxylic acids and the presence of $NaHCO_3$ was observed by Elsden [1938] and by Krebs & Eggleston [1940], who concluded that oxaloacetic acid is synthesized in animal tissues by condensation of pyruvic acid with CO_2. It is therefore suggestive to assume that succinic acid in yeast is formed in a similar way. The 'carboxylation' of pyruvic acid would represent a side reaction in the metabolism of pyruvate, the main reaction being the decarboxylation of pyruvic acid. In accordance with this view are the observations that oxaloacetate and malate increase the yield of succinate from glucose, and that yeast is able to bind CO_2 [Ruben & Kamen, 1940].

(3) *Formation of succinate from acetate.* According to Table 5 no succinate was formed from acetate (Na or Ba salt), contrary to the results of Wieland & Sonderhoff [1932], Sonderhoff & Deffner [1936], Sonderhoff & Thomas [1937]. It should be noted that our experimental conditions differ in some ways from those of Wieland and his collaborators. In our experiments fresh yeast, as opposed to 'impoverished' yeast, was used and the relatively short experimental times (24 hr.) excluded bacterial infection, which has previously been accepted by Wieland as an explanation of some of his results [Wieland *et al.* 1936].

Summary

1. The amount of succinate formed in resting suspensions of baker's yeast, brewer's yeast and *Torulopsis utilis* is proportional to the amount of sugar fermented. The yield of succinate varies between 1·35 and 3·96 g. mol. per 100 g. mol. of sugar. The quantity of succinate can exceed the dry weight of the fermenting yeast. This shows conclusively that most of the succinate formed during fermentation is derived from the fermented sugar. Ehrlich's view, according to which succinic acid is derived from the glutamic acid of autolysed yeast cells, is therefore to be discarded.

2. The formation of succinate depends on the presence of bicarbonate in the medium. No succinate is formed in 1 % KH_2PO_4; between 0·45 and 1·29 mg. succinate are formed when 50 mg. glucose are fermented by yeast suspended in 0·5 % $NaHCO_3$.

3. The amount of succinate formed from various fermentable sugars is of the same order.

4. Oxaloacetate, *l*-malate and *l*-aspartate increase the yield of succinate in the presence of glucose by about 20 %, α-ketoglutarate and α-hydroxyglutarate about 30 %, *l*-glutamate about 200 %, whilst various other substances had no effect.

5. No succinate is found when glucose is fermented by growing yeast cells.

6. Fumarase is absent from baker's yeast.

7. The mechanism of succinate formation from glucose is discussed.

The author wishes to express his gratitude to Dr H. A. Krebs for having suggested this investigation and for his constant help and advice, and also to the Sheffield Co-ordinating Committee for Refugees and to the International Student Service for a maintenance grant.

REFERENCES

Ehrlich (1909). *Biochem. Z.* **19**, 391.
Elsden (1938). *Biochem. J.* **32**, 187.
Fink & Krebs (1938). *Biochem. Z.* **299**, 1.
Krebs (1937). *Biochem. J.* **31**, 2095.
—— & Eggleston (1940). *Biochem. J.* **34**, 1383.
—— Smyth & Evans (1940). *Biochem. J.* **34**, 1041.
Neuberg & Ringer (1915). *Biochem. Z.* **71**, 226.
Pasteur (1860). *Ann. chim.* (*Phys.*), 3e série, **58**, 323.
Ruben & Kamen (1940). *Proc. Nat. Acad. Sci., Wash.*, **26**, 418.
Smyth (1940). *Biochem. J.* **34**, 1598.
Sonderhoff & Deffner (1936). *Liebigs Ann.* **525**, 132.
—— & Thomas (1937). *Liebigs Ann.* **530**, 195.
Stephenson & Yudkin (1936). *Biochem. J.* **30**, 506.
Wieland, Crawford & Walch (1936). *Liebigs Ann.* **525**, 119.
—— & Sonderhoff (1932). *Liebigs Ann.* **499**, 213.

52. NICOTINAMIDE, COZYMASE AND TISSUE METABOLISM

By P. J. G. MANN and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 1 April 1941*)

During the course of experiments on the respiratory activities of intact, minced and ground mammalian tissues we have found that there exists in brain and in liver a very active mechanism which accomplishes the breakdown of cozymase under both aerobic and anaerobic conditions. We have also found that this mechanism can be inhibited in a specific manner by nicotinamide. A preliminary note incorporating these results has already been published [Mann & Quastel, 1941]. The following communication is concerned with a description in detail of these and further results, together with observations on the effects of nicotinamide on the inhibitory activity of cobra venom in respiratory systems.

Technique

Measurements of respiratory activity

The Warburg apparatus has been used for the measurement of rates of O_2 uptake, or of CO_2 output when using the anaerobic technique of Quastel & Wheatley [1938]. The volume of fluid in the respiratory vessel was always maintained at 3 ml. and experiments were at 37° for periods usually not longer than 1 hr.

Preparation of biological material

Ground tissues. In our first experiments with ground brain, we used rat brain tissue (1 part brain to 10 parts 0·9 % NaCl) which had been finely ground in the wet-crushing mill of Booth & Green. This gave an excellent preparation having a high activity in breaking down cozymase. Subsequently, however, it was found unnecessary to use the wet-crushing mill, since a very active preparation could be made simply by grinding the tissue with sand in the presence of 0·9 % NaCl. The finely ground tissue was squeezed through muslin and the extract diluted with 0·9 % NaCl so that 10 ml. of the fluid contained 1 g. of the original tissue. This technique has been adopted for the preparation of ground tissues of rat brain, rat liver and rat kidney.

Muscle extract. Dialysed skeletal (rabbit) muscle extract as a source of cozymase-free lactic dehydrogenase was prepared by the method of Green *et al.* [1937].

Succinic and lactic dehydrogenases. The preparation made from washed pig's heart by the method of Keilin & Hartree [1938] sufficed as a source of both these enzymes. In some experiments extract of skeletal muscle or suspension of rat kidney was used as a lactic dehydrogenase.

Heart flavoprotein. This was prepared by a method similar to that described by Straub [1939].

Cozymase. A partially purified preparation, originally obtained from yeast, was used without further purification, as a high degree of purity was not required. Its activity was frequently tested by appropriate control experiments.

Media

All media in the aerobic experiments contained 0·02 *M* Na phosphate buffer *p*H 7·4 and all reagents were neutralized so that the *p*H of the fluid in the manometric vessel was 7·4. When tissue slices were investigated a phosphate-Locke medium was used. This contained 0·02 *M* Na phosphate *p*H 7·4, 0·004 *M* K^+ and 0·002 *M* Ca^{++}. An atmosphere of air was used in all aerobic experiments in which tissue slices were not involved; when intact slices were used air in the manometric vessels was displaced by O_2. In anaerobic experiments, using the ferricyanide technique, phosphate was omitted. The medium consisted of 0·025 *M* $NaHCO_3$ together with the other constituents under investigation. The atmosphere was 95 % N_2 + 5 % CO_2. 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was placed in the side tube and tipped into the main vessel after temperature equilibration. For further details of this technique see Quastel & Wheatley [1938].

In most experiments either 0·1 or 0·2 ml. of the cozymase solution was placed in the side tube of the manometric vessel, and after temperature equilibration had been attained this was tipped into the main vessel containing the tissue and respiratory constituents. Any variation in this procedure will be made clear in the text.

Results

The effects of addition of ground brain suspensions on the lactic dehydrogenase activities of muscle and kidney

The addition of small quantities of ground rat brain suspension to a lactic dehydrogenase system prepared from mammalian tissues results in a profound fall in the activity of the system. This is shown using systems prepared from rabbit skeletal muscle, ground pig heart muscle and ground rat kidney. With rabbit skeletal muscle a system is prepared similar to that used by Corran *et al.* [1939]. This consists of dialysed rabbit skeletal muscle extract (which is cell-free), pig heart flavoprotein, Na lactate, NaCN, methylene blue and cozymase. This system as shown by Corran *et al.* [1939] takes up O_2, pyruvate being formed and fixed by the cyanide. Hydrogen from the lactate passes eventually to O_2 through the chain of cozymase, flavoprotein and methylene blue. The addition to the system of 0·2 ml. of a rat brain suspension may reduce by 50 % the rate at which it consumes O_2. Typical results are shown in Table 1 and in Fig. 1. When a suspension of washed ground pig heart muscle is used as a source of lactic dehydrogenase there is no necessity to add flavoprotein as the suspension appears already to possess ample quantities. The feeble O_2 uptake secured by a mixture of washed ground pig heart muscle, Na lactate, NaCN and methylene blue is much increased by the addition of cozymase. The addition however of a mixture of cozymase and 0·2 ml. rat brain suspension reduces this rate of O_2 uptake to a value less than that which obtains in the absence of added cozymase (see Table 1). A ground rat kidney suspension provides a good source of a lactic dehydrogenase preparation relatively free from cozymase. Such a suspension, when used in small quantities, takes up but little O_2 in the presence of Na lactate, NaCN and methylene blue. The addition of cozymase powerfully stimulates the rate of O_2 uptake (Table 1, (3) and (4)). The further addition of a small quantity of ground rat brain suspension reduces the oxidative activity to very small values. This is illustrated by the results shown in Table 1.

Table 1. *Effects of ground brain suspension on lactic dehydrogenase activity*

Brain suspension was prepared by grinding finely 3·1 g. fresh rat brain in 31 ml. 0·9 % NaCl. Lactic dehydrogenase system consisted of lactic dehydrogenase preparation, pig heart flavoprotein (0·5 ml.), sodium lactate (0·13 *M*), sodium cyanide (0·08 *M*), phosphate buffer (0·02 *M*) *p*H 7·4, methylene blue and cozymase. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Cozymase mg.	Rat brain suspension ml.	μl. O_2 uptake
				In 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1	0	205·2
		1	0·2	108·9
		1	0·4	64·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	0	0	45·9
		2	0	136·2
		2	0·2*	28·5
(3) 0·2 ml. ground rat kidney suspension	0·04	0	0	33·5
		2	0	179·5
				In 30 min.
(4) 0·2 ml. ground rat kidney suspension	0·04	0	0	30·1
		1	0	66·0
		1	0·2	10·5

* In this experiment the cozymase and rat brain suspension were incubated together for 15 min. at 37° before adding to the main system.

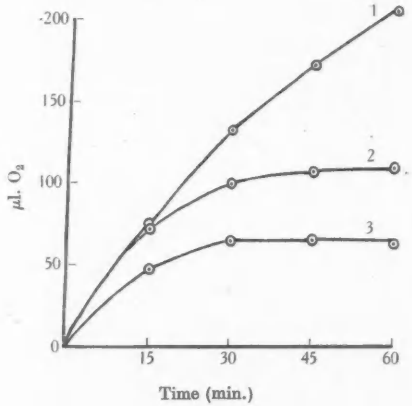


Fig. 1. Effects of ground brain suspensions on oxygen uptake by lactic dehydrogenase system (muscle extract, flavoprotein, lactate, cyanide, cozymase and methylene blue).

1. Lactic dehydrogenase system.
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·4 ml. ground rat brain suspension.

Destruction of cozymase by suspensions of ground mammalian tissues

The powerful inhibitory effects of ground brain suspensions on lactic dehydrogenase systems are due to their destruction of cozymase. This is proved by the following facts:

(1) When cozymase is added to a system containing lactic dehydrogenase, a ground brain suspension, lactate, cyanide and methylene blue, the inhibition of

the rate of O_2 uptake due to the brain develops slowly, the rate of development of the inhibition depending on the amount of brain used. Thus, as seen in Table 1 (1) and in Fig. 1, the presence of 0·4 ml. rat brain suspension brings about a much larger inhibition of the rate of O_2 uptake than occurs in the presence of 0·2 ml. brain suspension. If, however, the cozymase is incubated together with 0·2 ml. brain suspension for 15 min. at 37°, before addition to the mixture of lactic dehydrogenase, lactate, cyanide and methylene blue, a large inhibition of the rate of O_2 uptake takes place at once—the mixture behaving in fact as though no cozymase had been added. This is shown by the results given in Table 2. It is clear from these results that both rat brain and rat liver are able

Table 2. *Destruction of cozymase by tissues*

Lactic dehydrogenase system and brain suspension as in Table 1. Liver and kidney suspensions made by grinding tissue with sand and saline (1 g. tissue to 10 ml. saline) and squeezing through muslin. Air; 37°.

Source of lactic dehydrogenase	Methylene blue %	Treatment of cozymase	μl. O_2 uptake in 1 hr.
(1) 0·3 ml. rabbit skeletal muscle extract	0·017	1 mg. added. Untreated	262·8
		1 mg. incubated with 0·2 ml. brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	44·3
		1 mg. incubated in absence of brain suspension for 15 min. at 37° before addition to lactic dehydrogenase system	272·0
(2) 0·3 ml. washed ground pig heart muscle	0·04	1 mg. added. Untreated	136·2
		1 mg. incubated with 0·2 ml. rat kidney suspension for 15 min. at 37° before addition to lactic dehydrogenase system	243·6
(3) 0·3 ml. rat kidney suspension	0·04	No cozymase added	59·9
		2 mg. added. Untreated	189·4
		2 mg. incubated with 0·2 ml. liver suspension for 15 min. at 37° before addition to lactic dehydrogenase system	57·8

to destroy cozymase. However, after the addition of an incubated mixture of rat kidney suspension and cozymase to the lactic dehydrogenase of pig heart, an acceleration instead of an inhibition of O_2 uptake takes place. This is due to the fact that rat kidney has but little ability to destroy cozymase and also provides an extra source of lactic dehydrogenase.

(2) The inhibition of the lactic dehydrogenase-cozymase system by a brain suspension takes place anaerobically, using ferricyanide as oxidant of reduced cozymase instead of flavoprotein and methylene blue (Table 3). This result shows that the inhibition cannot be attributed to a destruction of flavoprotein by the brain suspension.

(3) The inhibitory effect of cozymase on succinic dehydrogenase activity [Keilin & Hartree, 1940] is entirely neutralized by the addition to the system of a small quantity of brain suspension. This result is shown in Table 12 and will be referred to in more detail later.

Anaerobic destruction of cozymase by brain

Cozymase is broken down by a brain suspension under strictly anaerobic conditions. The experiment demonstrating this is carried out by using a Warburg manometric vessel equipped with two side arms. In one side arm is placed the brain suspension, in the other ferricyanide solution. The main vessel contains a lactic dehydrogenase preparation, cozymase, Na lactate, NaCN and $NaHCO_3$ (0·025 *M*). The air in the apparatus is displaced by 95 % N_2 + 5 % CO_2, and only after strictly anaerobic conditions are obtained is the brain suspension tipped into the main vessel containing the cozymase. Finally, after temperature equilibration, the ferricyanide is tipped into the main vessel. A control experiment, with no brain suspension present, is also carried out. As the results given in Table 3 show, the effect of the brain suspension under strictly anaerobic conditions is to reduce the activity of the lactic dehydrogenase system.

Table 3. *Anaerobic destruction of cozymase by brain*

Rat kidney suspension was used as source of lactic dehydrogenase. Brain suspension was made up as in Table 1.

Medium. Bicarbonate (0·025 *M*)-saline, Na lactate 0·13 *M*; NaCN 0·08 *M*; 1 mg. cozymase. 95 % N_2 + 5 % CO_2; 37°. Vessels with two side arms were used:

In 1st vessel: 0·2 ml. brain suspension placed in one side arm.
0·2 ml. 8·3 % $Na_3Fe(CN)_6$ placed in other side arm.
Lactic dehydrogenase system in main vessel.

In 2nd vessel: As above, but no brain suspension present.

After anaerobic conditions were secured, the brain suspension was added to the lactic dehydrogenase system in first vessel. After equilibration, the ferricyanide was added to both vessels.

	μl. CO_2 output in 1 hr.*
Lactic dehydrogenase system; no brain suspension added	378·8
Lactic dehydrogenase system; 0·2 ml. brain suspension added	204·9

* Retention of CO_2 due to addition of small volume of brain suspension is here neglected.

This result shows that the destruction of cozymase by the brain suspension is not due to an oxidative breakdown and we conclude, for the time being, that the destruction of the coenzyme is accomplished by a hydrolytic enzyme. We will refer to this as cozymase nucleotidase.

Table 4. *Thermolability of cozymase nucleotidase*

Lactic dehydrogenase system: Washed ground pig heart suspension (0·5 ml.), 0·13 *M* Na lactate; 0·02 *M* phosphate buffer *p*H 7·4; 0·08 *M* NaCN; 0·04 % methylene blue. 1 mg. cozymase. Ground brain suspension used as cozymase nucleotidase. Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system, no cozymase added	34·1
Lactic dehydrogenase system, with cozymase	183·0
Lactic dehydrogenase system + cozymase previously incubated with 0·2 ml. brain suspension for 15 min. at 37°	38·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain, which had been heated for 5 min. at 70°	161·3
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. brain which had been heated for 5 min. at 100°	173·4

Thermolability of cozymase nucleotidase

Results given in Table 4 show that the enzyme in brain responsible for the breakdown of cozymase is thermolabile. It is completely destroyed by exposure

to 100° for 5 min. and almost completely destroyed by exposure to 70° for the same period.

Can the inactivation of cozymase be due to preferential adsorption on brain proteins?

The possibility arises [cf. Lipton & Elvehjem, 1940] that the inactivation of cozymase by a brain extract may not be due to hydrolysis of the coenzyme but to its preferential adsorption on, or combination with, brain proteins. According to this suggestion the coenzyme is not destroyed, but removed from its sphere of action by adsorption on proteins having a high affinity for it.

This problem has been attacked in the following way. Cozymase is incubated together with a brain suspension at 37° for 15 min., when the inactivation of cozymase is complete. The mixture is divided into lots *A*, *B* and *C*. Mixture *A* is left undisturbed. Mixture *B* is heated for 5 min. at 70°. This treatment is known to destroy the inactivating mechanism and presumably, therefore, the proteins responsible for adsorption of the cozymase. Since the cozymase, by hypothesis, is not broken down by adsorption on brain proteins, it should be liberated from the heat-treated proteins. Hence mixture *B*, in contrast to mixture *A*, should contain free cozymase. Mixture *C* is heated for 5 min. at 70° and to this, after cooling, is added free cozymase. If the brain proteins, alleged to be responsible for the adsorption of cozymase, are destroyed by the heat treatment, they will now be unable to inactivate the added cozymase. Hence it should follow if the adsorption theory be correct, that mixture *A* will contain no free cozymase, and that mixtures *B* and *C* will contain free cozymase, *C* being richer in this respect than *B*. On the other hand if the adsorption theory be incorrect, both mixtures *A* and *B* will contain no free cozymase whilst mixture *C* will contain the free cozymase which was subsequently added to it.

Experiment (see Table 5) shows that no free cozymase is liberated from the incubated cozymase-brain mixture after heat treatment and that the heated mixture is unable to inactivate any added cozymase. These facts are in accordance with the view that cozymase is inactivated by breakdown by an enzyme in the brain and not by adsorption on special proteins.

Table 5. *Effect of heating cozymase-brain extract mixture after incubation*

The following mixture was prepared: Ground rat brain suspension (0·6 ml.), cozymase (3 mg.) in 2·4 ml. phosphate buffer (0·02*M*) *p*H 7·4. This was incubated at 37° for 15 min. and divided into three equal lots (*A*, *B*, *C*).

A was not treated further.

B was heated for 5 min. at 70° and cooled.

C was heated for 5 min. at 70°, cooled and to it was added 1 mg. cozymase.

A, *B* and *C* were transferred to Warburg vessels containing a lactic dehydrogenase system (washed ground pig's heart suspension 0·5 ml., Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·04 % and 0·02*M* phosphate buffer *p*H 7·4). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system containing *A*	36·2
Lactic dehydrogenase system containing *B*	32·2
Lactic dehydrogenase system containing *C*	193·2

Effects of addition of nicotinamide

The addition of nicotinamide to a ground brain suspension has the effect of greatly inhibiting the cozymase nucleotidase. This is shown most clearly by incubating a mixture of brain suspension, cozymase and nicotinamide for

15 min. at 37° and adding the mixture, after incubation, to a lactic dehydrogenase preparation in presence of lactate, cyanide and methylene blue. The effect of the nicotinamide is to prevent almost completely the breakdown of the cozymase.

Typical results with (*a*) the lactic dehydrogenase of a dialysed rabbit skeletal muscle extract and with (*b*) the lactic dehydrogenase of a ground rat kidney suspension are shown in Tables 6 and 7 respectively.

Table 6. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (0·5 ml.), pig heart flavoprotein (0·5 ml.), Na phosphate buffer 0·02*M* *p*H 7·4, Na lactate 0·13*M*, NaCN 0·08*M*, methylene blue 0·017 %. Cozymase (1 mg.) was incubated at 37° for 15 min. with 0·2 ml. ground rat brain suspension in phosphate buffer *p*H 7·4 in the presence and absence of nicotinamide. The mixture was then added to the lactic dehydrogenase system, and O_2 consumption measured. Air; 37°.

	μl. O_2 uptake in 1 hr.
1. Lactic dehydrogenase system with untreated cozymase	215·4
2. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension	54·1
3. Lactic dehydrogenase system with cozymase previously incubated for 15 min. with 0·2 ml. brain suspension in presence of 0·3 ml. 1 % nicotinamide	167·2
4. As in 3 but using 0·1 ml. 10 % nicotinamide	199·4
5. As in 3 but using 0·3 ml. 10 % nicotinamide	171·3
6. Lactic dehydrogenase system with untreated cozymase and with 0·3 ml. 1 % nicotinamide present (i.e. final concentration of nicotinamide =0·1 %)	195·9

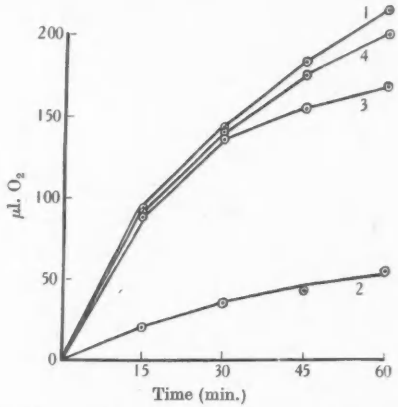


Fig. 2. Inhibitory action of nicotinamide on cozymase nucleotidase.

1. Lactic dehydrogenase system (muscle extract, etc.).
2. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension.
3. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·1 % nicotinamide.
4. Lactic dehydrogenase system + 0·2 ml. ground rat brain suspension previously incubated with 0·3 % nicotinamide.

Examination of the results given in Table 6 and in Fig. 2 shows that the protective effect of nicotinamide increases with its concentration, but that above an

optimal concentration the nicotinamide inhibits the O_2 uptake. Probably this small inhibitory effect is that already described by Baker *et al.* [1938]. This effect has not yet been explained but it seems likely that it is due to competition of nicotinamide with cozymase for lactic dehydrogenase.

The presence of nicotinamide increases the O_2 uptake of the kidney lactic dehydrogenase system, containing added cozymase even in the absence of brain suspension. This points to the presence in the ground kidney suspension of a small quantity of cozymase nucleotidase. The very powerful inhibitory effect of a brain suspension on the kidney system is almost completely neutralized by the presence of 0·1 % nicotinamide (Table 7).

Table 7. *Effects of addition of nicotinamide aerobically*

Lactic dehydrogenase system: Rat kidney suspension (prepared by grinding fresh kidney in saline, 1 g. to 10 ml., with sand and squeezing through muslin); phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %. 0·2 ml. of the kidney suspension was placed in side tube of Warburg vessel and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system	30·1
Kidney lactic dehydrogenase system + cozymase (1 mg.)	66·0
Kidney lactic dehydrogenase system + cozymase + 0·1 % nicotinamide	89·8
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension	10·5
Kidney lactic dehydrogenase system + cozymase (1 mg.) + 0·2 ml. ground brain suspension + 0·1 % nicotinamide	77·1

Cozymase is not only broken down by ground brain suspensions but by intact brain slices, this effect being inhibited by the presence of nicotinamide. These results are shown in Table 8. The destruction of cozymase by the intact brain slices is only partially due to cozymase nucleotidase diffusing from the tissue

Table 8. *Effect of nicotinamide on cozymase in brain slices*

Equal weights (100 mg. wet wt.) of rat brain slices were placed in Warburg vessels containing the following media:

A 0·02*M* Na phosphate *p*H 7·4, Locke solution, 2 mg. cozymase preparation.
B As *A* but containing also 0·3 % nicotinamide.
C 0·02*M* Na phosphate *p*H 7·4, Locke solution.
D As *C* but containing also 0·3 % nicotinamide.

In another vessel was placed medium *E*: 0·02*M* Na phosphate *p*H 7·4, Locke solution and 2 mg. cozymase preparation (no tissue slices present). These were incubated at 37° in O_2 for 30 min., after which slices of tissue were removed from all vessels. To *C* and *D* were then added 2 mg. cozymase preparation and these vessels were incubated for a further 30 min. in O_2 at 37°.

1 ml. of each of the above media, i.e. *A*, *B*, *C*, *D*, *E*, was then transferred to fresh Warburg vessels, containing 0·02*M* Na phosphate buffer *p*H 7·4, 0·13*M* Na lactate, 0·08*M* NaCN, 0·017 % methylene blue, and 0·16 % nicotinamide. In the side tube of each vessel was placed 0·3 ml. suspension of ground rat kidney (1 g. to 10 ml. saline). After temperature equilibration (37°) in air, the kidney suspension was tipped in the main vessels and rates of O_2 uptakes were measured.

	μl. O_2 uptake in 30 min.
Kidney lactic dehydrogenase system + 1 ml. *A*	36·4
Kidney lactic dehydrogenase system + 1 ml. *B*	112·5
Kidney lactic dehydrogenase system + 1 ml. *C*	88·7
Kidney lactic dehydrogenase system + 1 ml. *D*	131·4
Kidney lactic dehydrogenase system + 1 ml. *E*	140·4

slices in the outer medium. Much of the cozymase must be destroyed by cozymase nucleotidase within the intact tissue. The intact brain slices must, clearly, be permeable to cozymase, a conclusion already arrived at by Quastel & Wheatley [1938].

The protective effect of nicotinamide on cozymase breakdown may be demonstrated also by experiments carried out under anaerobic conditions.

If a brain suspension is, itself, used as a source of lactic dehydrogenase it may be shown that the addition of cozymase to it has but little effect on its power of reducing ferricyanide under anaerobic conditions. The addition of nicotinamide also (in the absence of added cozymase) has little or no effect on the reduction of ferricyanide. If, however, a mixture of cozymase and nicotinamide be added to the brain suspension, a powerful reduction of ferricyanide ensues. These results are shown in Tables 9 and 10. The results are easily explained by the fact that the very active cozymase nucleotidase of brain quickly

Table 9. *Effects of nicotinamide anaerobically*

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. saline. 1 ml. brain suspension was placed in each Warburg vessel containing the following medium: $NaHCO_3$ 0·025*M*, NaCl 0·13*M*, Na lactate and 0·05*M* NaCN. Nicotinamide (0·3 %) or cozymase (1 mg.) or both were present in certain vessels. After removal of air by 95 % N_2 + 5 % CO_2, and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped from side tube into main vessel.

	μl. CO_2 output in 1 hr.
Brain + lactate system	124·4
Brain + lactate system + nicotinamide	129·5
Brain + lactate system + cozymase	136·6
Brain + lactate system + cozymase + nicotinamide	341·0

Table 10. *Effects of addition of nicotinamide anaerobically*

One half (0·6 g.) of a fresh rat brain was ground in 6 ml. Locke solution. The other half of the rat brain was ground in 6 ml. Locke solution containing 3 % nicotinamide. 1 ml. of each of these suspensions was placed in a $NaHCO_3$ (0·025*M*)-Locke medium containing 0·13*M* Na lactate, 0·05*M* NaCN, with and without the addition of cozymase (1 mg.). After removal of air by 95 % N_2 + 5 % CO_2 and equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into each vessel.

	μl. CO_2 output in 1 hr.
Brain ground in Locke solution only. No cozymase added	168·9
Brain ground in Locke solution only. Cozymase added	185·7
Brain ground in Locke solution containing nicotinamide. No cozymase added	149·6
Brain ground in Locke solution containing nicotinamide. Cozymase added	387·4

destroys all free cozymase,[1] and not until the enzyme is inhibited by nicotinamide can the addition of free cozymase show its normal catalytic effect on lactic dehydrogenase activity. The results given in Table 10 show the stimulating effect of cozymase on the reducing power of brain ground in a nicotinamide Locke solution as compared with that ground in Locke solution free from nicotinamide.

Can the apparent protective effect of nicotinamide be due to synthesis to cozymase?

A possible explanation of the effect of nicotinamide in stimulating the O_2 uptake under the given experimental conditions is that the nicotinamide is built up into free cozymase.

[1] We have evidence to show that brain tissue contains a small quantity of bound cozymase which is broken down to produce free cozymase on heating the tissue.

If this view is true the apparent protective effect of nicotinamide on cozymase breakdown by a brain suspension is not in fact due to a competition of nicotinamide with cozymase for a cozymase nucleotidase, but to the synthesis of cozymase by the brain suspension from nicotinamide and other constituents present in the brain suspension. It may be shown, however, that 0·3 % nicotinamide which will apparently prevent the breakdown of cozymase by a brain suspension gives rise to little or no free cozymase when incubated alone with the brain suspension. These results are shown in Table 11 and indicate that a synthesis of cozymase from nicotinamide under the experimental conditions

Table 11. *Test for synthesis of cozymase*

Lactic dehydrogenase system: Washed ground pig's heart suspension (0·3 ml.); Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %.

Cozymase (1 mg.) or rat brain suspension or both, in the presence or absence of 0·1 ml. 10 % nicotinamide, were incubated aerobically in Warburg vessels at 37° for 15 min. The lactic dehydrogenase system was then added to each vessel and the O_2 consumption was measured. Air; 37°.

	μl. O_2 uptake in 30 min.
Lactic dehydrogenase system + cozymase	194·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension	10·5
Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	164·5
Lactic dehydrogenase system + 0·2 ml. ground brain suspension	9·7
Lactic dehydrogenase system + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	14·7

employed does not take place. Hence the alternative explanation, that nicotinamide competes with cozymase for a cozymase nucleotidase is probably the correct one. This is supported by the result quoted in Table 10 in which it is shown that the addition of nicotinamide alone to minced brain does not increase its lactic dehydrogenase activity. Moreover, it may be shown (see Fig. 2) that the effect of nicotinamide in increasing the O_2 uptake of a lactic dehydrogenase system containing cozymase and brain suspension falls off with time, whereas if synthesis of cozymase took place, the effect of nicotinamide should increase with time.

Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase

It is known [Potter, 1939; Keilin & Hartree, 1940] that the addition of cozymase to a suspension of washed ground pig's heart greatly reduces the latter's succinic dehydrogenase activity. This is attributed by Keilin & Hartree to the formation of oxaloacetate from succinate by the action of succinic dehydrogenase and of malate dehydrogenase in conjunction with cozymase. The oxaloacetate formed, even in minute quantities, inhibits succinic dehydrogenase.

It may be shown that the addition of a ground brain suspension entirely removes the inhibitory effect exercised by cozymase on the succinic dehydrogenase activity of pig's heart. If nicotinamide, however, be added at the same time as the ground brain suspension, the cozymase exercises its normal inhibitory effect. These results are shown in Table 12, and are explained satisfactorily by the presence in brain of a cozymase nucleotidase which breaks down cozymase and whose activity is inhibited by the presence of nicotinamide.

It may also be shown that cozymase inhibits the anaerobic oxidation of succinate by ferricyanide in the presence of a brain suspension acting as sole source of succinic dehydrogenase. This effect is greatly increased by the addition

Table 12. *Effects of nicotinamide on succinic dehydrogenase activity in presence and absence of cozymase*

Succinic dehydrogenase system: Washed ground pig's heart suspension (1 ml.), Na succinate 0·03 *M*; phosphate buffer (0·02 *M*) *p*H 7·4. Air; 37°.

	μl. O_2 uptake in 1 hr.
Succinic dehydrogenase system	241·2
Succinic dehydrogenase system + 0·3 % nicotinamide	253·5
Succinic dehydrogenase system + cozymase (1 mg.)	119·8
Succinic dehydrogenase system + cozymase (1 mg.) + 0·3 % nicotinamide	123·9
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension	270·0
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	272·4
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + cozymase (1 mg.)	236·5
Succinic dehydrogenase system + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide + cozymase (1 mg.)	130·8

to the system of nicotinamide. These results are shown in Table 13. The phenomena are explained by the presence in the brain suspension of cozymase nucleotidase. The inhibitory action of added cozymase on the succinic dehydrogenase of brain is determined by the amount of free cozymase present, and by the speed with which it can form oxaloacetic acid as compared with the speed with which it is broken down by the cozymase nucleotidase. In the presence of nicotinamide the breakdown of cozymase by the brain is inhibited and hence more free cozymase is available to build up oxaloacetic acid.

Table 13. *Effects of nicotinamide on succinic dehydrogenase activity of brain under anaerobic conditions*

Medium: $NaHCO_3$ (0·025 *M*)-saline. 95 % N_2 + 5 % CO_2; 37°. After equilibration 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel.

	μl. CO_2 output in 30 min
1 ml. ground rat brain suspension	12·0
1 ml. ground rat brain suspension + 1·0 % nicotinamide	13·7
1 ml. ground rat brain suspension + Na succinate 0·03 *M*	337·6
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + 1·0 % nicotinamide	266·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.)	210·9
1 ml. ground rat brain suspension + Na succinate 0·03 *M* + cozymase (1 mg.) + 1·0 % nicotinamide	128·0

Keilin & Hartree [1940] could find no inhibitory effect of cozymase on the succinic dehydrogenase of brain and attributed this apparent inactivity of cozymase to the rapid breakdown of oxaloacetic acid by brain. This explanation cannot be wholly correct as the results in Table 13 show that the presence of cozymase can inhibit succinic dehydrogenase of brain and hence that the breakdown of oxaloacetic acid by brain is not sufficiently rapid to prevent the inhibition taking place. It is more likely that the failure of Keilin & Hartree to observe an inhibitory effect of cozymase on brain succinic dehydrogenase is due to the rapid destruction of the cozymase by the brain preparation under their experimental conditions.

Effects of Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide

Chain [1939] has shown that cozymase is destroyed by small quantities of cobra venom. This phenomenon we have confirmed. The inhibitory effect of the venom on the lactic dehydrogenase-cozymase system of pig heart is neutralized

by the addition of nicotinamide. 0·3 % nicotinamide neutralizes almost completely the inhibitory effect of 0·5 mg. dried cobra venom. The neutralizing effect of the nicotinamide increases with increase of concentration of nicotinamide and decreases with increase of concentration of the venom. This points to the existence of an equilibrium between nicotinamide and an enzyme in the cobra venom. Whether this enzyme is identical with the cozymase nucleotidase of brain and liver remains to be seen. Results illustrating these phenomena are shown in Table 14.

Table 14. *Effects of dried Indian cobra venom on lactic dehydrogenase activity in presence and absence of nicotinamide*

Lactic dehydrogenase system: Washed ground pig's heart suspension (1 ml.); Na phosphate buffer 0·02*M* pH 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %; cozymase 1 mg. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
Lactic dehydrogenase system	164·8
Lactic dehydrogenase system + 0·5 mg. venom	95·2
Lactic dehydrogenase system + 0·5 mg. venom + 0·3 % nicotinamide	154·3
Lactic dehydrogenase system + 0·75 mg. venom	50·1
Lactic dehydrogenase system + 0·75 mg. venom + 0·3 % nicotinamide	99·1
Lactic dehydrogenase system + 1 mg. venom	36·2
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	77·0
Lactic dehydrogenase system + 0·3 % nicotinamide	163·6
Exp. 2.	
Lactic dehydrogenase system	123·0
Lactic dehydrogenase system + 1 mg. venom	52·9
Lactic dehydrogenase system + 1 mg. venom + 0·03 % nicotinamide	53·0
Lactic dehydrogenase system + 1 mg. venom + 0·1 % nicotinamide	73·0
Lactic dehydrogenase system + 1 mg. venom + 0·3 % nicotinamide	92·4

Specificity of nicotinamide

Nicotinic acid will not replace nicotinamide as an inhibitor of the breakdown of cozymase by a rat brain suspension. Adenylic acid appears to have a feeble effect in protecting cozymase from breakdown. Propionamide and coramine are without action. These results are given in Table 15 and illustrate the specificity of action of nicotinamide.

Neither NaF nor $Na_4P_2O_7$ at the concentrations tried (see Table 16) prevents the breakdown of cozymase by a brain suspension.

Malate dehydrogenase and nicotinamide

Observations recorded in Table 17 show that substantially the same conclusions which apply to the lactic dehydrogenase system apply also to the malate dehydrogenase system. This is shown using the malate dehydrogenase of rat kidney under aerobic conditions and that of rat brain under anaerobic conditions. In the former case the addition of a suspension of rat brain effects an inhibition of malate oxidation which is neutralized by the presence of nicotinamide. In the latter case the presence of nicotinamide greatly increases the anaerobic oxidation of *dl*-malate in the presence of brain by ferricyanide.

Table 15. *Specificity of nicotinamide*

Lactic dehydrogenase system: 0·3 ml. ground rat kidney suspension; Na phosphate buffer 0·02*M* *p*H 7·4; Na lactate 0·13*M*; NaCN 0·08*M*; methylene blue 0·04 %. The rat kidney suspension was placed in the side tube of the vessel and tipped into the main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Exp. 1.	
1. Lactic dehydrogenase system	31·7
2. Lactic dehydrogenase system + cozymase (2 mg.)	139·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	40·3
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinamide	158·6
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % nicotinic acid (Na salt)	41·8
6. As in 5 but using 1 % nicotinic acid (Na salt)	38·4
7. As in 5 but using 0·1 % nicotinic acid (Na salt)	47·7
8. As in 5 but using 0·03 % nicotinic acid (Na salt)	45·1
Exp. 2.	
1. Lactic dehydrogenase system	44·2
2. Lactic dehydrogenase system + cozymase (2 mg.)	257·0
3. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	64·6
4. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	268·5
5. Lactic dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 10 mg. adenylic acid (muscle)	75·7
6. As in 5 but using 10 mg. yeast adenylic acid	80·9
7. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (muscle)	208·4
8. Lactic dehydrogenase system + cozymase + 10 mg. adenylic acid (yeast)	224·0
9. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·3 % propionamide	57·3
10. Lactic dehydrogenase system + cozymase + 0·2 ml. ground brain suspension + 0·1 % coramine	62·5
11. As in 10 but using 0·3 % coramine	53·4

Table 16. *Actions of fluoride and of pyrophosphate*

Lactic dehydrogenase system: Rabbit skeletal muscle extract (1 ml.), pig's heart flavoprotein, Na lactate (0·13*M*); NaCN (0·08*M*); methylene blue (0·04 %); phosphate buffer 0·02*M* *p*H 7·4 (see also Table 6). Air; 37°.

	μl. O_2 uptake in 1 hr.
Lactic dehydrogenase system + cozymase (1 mg.)	202·9
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension	44·0
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·1*M* NaF	49·8
Lactic dehydrogenase system + cozymase previously incubated for 15 min. at 37° with 0·2 ml. ground rat brain suspension in presence of 0·04*M* $Na_4P_2O_7$	39·2

Table 17. *Malate dehydrogenase and nicotinamide*

Aerobic

Malate dehydrogenase system: 0·3 ml. ground rat kidney suspension, Na phosphate buffer 0·02*M* *p*H 7·4; Na *dl*-malate (0·017*M*); NaCN 0·08*M*; methylene blue 0·04 %. The kidney suspension was placed in the side tube and tipped into main vessel after equilibration. Air; 37°.

	μl. O_2 uptake in 1 hr.
Malate dehydrogenase system + cozymase (2 mg.)	96·4
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension	26·6
Malate dehydrogenase system + cozymase + 0·2 ml. ground rat brain suspension + 0·3 % nicotinamide	92·5

Anaerobic

One rat brain (1·6 g.) was finely chopped and suspended in 16 ml. Locke solution. 1 ml. suspension was placed in the following medium: $NaHCO_3$ (0·025*M*)-saline, NaCN 0·05*M*, with or without Na *dl*-malate (0·017*M*), cozymase (1 mg.), and nicotinamide. After removal of air by 95 % N_2 + 5 % CO_2, and after equilibration at 37°, 0·2 ml. 8·3 % $Na_3Fe(CN)_6$ was tipped into main vessel from side tube.

	μl. CO_2 output in 1 hr.
Brain system	85·6
Brain system + 1 % nicotinamide	85·8
Brain system + *dl*-malate	111·0
Brain system + *dl*-malate + 1 % nicotinamide	198·1

Discussion

It is evident that brain, both in the intact and ground condition, is able to break down cozymase at a rapid rate. The enzyme involved is present also in liver and to a small extent in kidney. It appears to be absent from a dialysed extract of rabbit skeletal muscle or from washed ground pig's heart. Possibly the same enzyme is present in cobra venom. It may also be present in tumours [cf. Boyland & Boyland, 1935] and in yeast for Leloir & Dixon [1937] have reported that crushed yeast is able to destroy cozymase.

Since brain contains a very active cozymase nucleotidase, it must be able to synthesize cozymase rapidly *in vitro* as well as *in vivo* in order to balance the nucleotidase activity and to account for the maintenance of oxidative activity of brain when this respires in lactate media. Alternatively it must be concluded that the cozymase nucleotidase of intact brain is ineffective under aerobic conditions—a conclusion which is incorrect as the foregoing observations have shown. There must therefore exist in intact brain an equilibrium between the mechanisms responsible for the synthesis of cozymase and those responsible for its breakdown. The existence of this equilibrium probably accounts, at least partially, for the fact that the respiration of damaged tissues is often much lower than that of intact tissues. In the former case damage to the tissues may impair synthetic mechanisms by alterations of cell permeability with loss of essential cell constituents by diffusion etc.

The high rates of breakdown of cozymase by brain or liver throw doubt on estimations of cozymase in tissue extracts where precautions have not been taken to destroy at once the cozymase nucleotidase. It is obviously essential that such destruction should be brought about immediately after dissection of the organ under investigation from the animal.

The presence of nicotinamide inhibits the breakdown of cozymase probably by competition with cozymase for the nucleotidase. The evidence shows that

the stimulating action of nicotinamide is not due to synthesis to cozymase under the given experimental conditions.

The demonstration of the action of nicotinamide in protecting cozymase from breakdown by mammalian tissues raises the question as to whether the well-known therapeutic effects of nicotinamide may not be due partly to this action. If this view is true, substances other than nicotinamide having a similar protective effect may have similar therapeutic effects. It may be noted in this connexion that Vilter & Spies [1939] have shown that quinolinic acid will cure pellagra and that Woolley *et al.* [1938] find that a variety of nicotinic acid derivatives will cure canine black tongue. Moreover, as a result of the administration of quinolinic acid, there has been found [Vilter & Spies, 1939] an increase in concentration of cozymase in the blood. It is clear that further investigation is required of the types of molecules which will inhibit cozymase breakdown by mammalian tissues.

Summary

1. The addition of a ground rat brain suspension to a lactic dehydrogenase-cozymase system or to a malic dehydrogenase-cozymase system of mammalian tissues brings about considerable diminution of the activity of the systems.

2. This is due to the destruction of cozymase by a mechanism present in brain. The evidence points to the mechanism in question being a hydrolytic enzyme which is referred to as cozymase-nucleotidase.

3. Cozymase-nucleotidase occurs in rat brain and in rat liver and to a relatively small extent in rat kidney. It is apparently absent from dialysed rabbit skeletal muscle extract and from washed ground pig's heart.

4. Cozymase nucleotidase is destroyed by heating at 70° for 5 min.

5. Evidence is given showing that cozymase is not inactivated by adsorption upon special proteins in brain.

6. The presence of nicotinamide greatly inhibits cozymase nucleotidase of brain. This may be shown by experiments carried out under aerobic and anaerobic conditions. Nicotinamide stimulates lactic and malic dehydrogenase activities of brain in presence of cozymase.

7. The stimulating effect of nicotinamide on the lactic dehydrogenase activities of tissues in presence of cozymase-nucleotidase is not due to a synthesis of nicotinamide to cozymase, but to a competition of nicotinamide with cozymase for the nucleotidase.

8. Intact brain slices break down cozymase, this effect being neutralized by the presence of nicotinamide. An equilibrium must exist in intact brain between a synthetic mechanism leading to cozymase formation and a mechanism leading to its breakdown.

9. The inhibition of succinic dehydrogenase of pig heart by cozymase is neutralized by the addition to the system of a ground brain suspension. The effect of the brain may be eliminated by the addition to the system of nicotinamide. The action of the nicotinamide here is to prevent the breakdown of the cozymase. Succinic dehydrogenase activity of a brain suspension, when examined anaerobically by the ferricyanide technique, is decreased by the addition of cozymase, the effect being enhanced by the simultaneous addition of nicotinamide. Here again the effect is due to the prevention of breakdown of cozymase by nicotinamide.

10. The action of cobra venom in inhibiting a lactic dehydrogenase-cozymase system by destruction of cozymase is retarded by nicotinamide. An equilibrium probably exists between nicotinamide and a nucleotidase in cobra venom.

11. The protective effect of nicotinamide on cozymase breakdown is not shown by nicotinic acid, coramine, propionamide, sodium fluoride or sodium pyrophosphate. Adenylic acid (of yeast or muscle) has a feeble protective action.

12. Attention is drawn to the possibility that the therapeutic effects of nicotinamide may be due partly to its protective effects on cozymase breakdown.

Our sincere thanks are due to the Rockefeller Foundation and to the Medical Research Council for their financial help, and to the Ella Sachs Plotz Foundation for a grant for the purchase of animals. We are also much indebted to Dr W. Feldberg for a supply of dried Indian cobra venom and to Dr P. Ostern for a sample of muscle adenylic acid.

REFERENCES

Baker, Fazekas & Himwich (1938). *J. biol. Chem.* **125**, 545.
Boyland & Boyland (1935). *Biochem. J.* **29**, 1097.
Chain (1939). *Biochem. J.* **33**, 407.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2329.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1940). *Proc. roy. Soc.* B, **129**, 277.
Leloir & Dixon (1937). *Enzymologia*, **2**, 81.
Lipton & Elvehjem (1940). *J. biol. Chem.* **136**, 637.
Mann & Quastel (1941). *Nature, Lond.*, **147**, 326.
Potter (1939). *Ark. Kemi Min. Geol.* **13** B, No. 7.
Quastel & Wheatley (1938). *Biochem. J.* **32**, 936.
Straub (1939). *Biochem. J.* **33**, 787.
Vilter & Spies (1939). *Lancet*, **2**, 423.
Woolley, Strong, Madden & Elvehjem (1938). *J. biol. Chem.* **124**, 715.

53. THE SITE OF ACTION OF NARCOTICS IN RESPIRATORY PROCESSES

By M. MICHAELIS and J. H. QUASTEL

From the Biochemical Laboratory, Cardiff City Mental Hospital

(*Received 7 March 1941*)

Whilst it is well known that narcotics as a general rule inhibit respiratory processes, their effects being mostly confined to the dehydrogenases, little is known as yet of the mechanism by which narcotics, when used at the small concentrations which have biological significance, exert their inhibitory effects.

It has been shown [Quastel & Wheatley, 1932; Jowett & Quastel, 1937; Jowett, 1938] that narcotics, such as chloretone and the barbiturates, greatly reduce the oxygen uptake of brain tissue (whether in the form of a mince or of tissue slices), as well as that of other organs, when the tissue respires in media containing glucose, sodium lactate or sodium pyruvate. Definite inhibitions of oxidative processes occur at concentrations of narcotics which bring about deep narcosis, the inhibitions being reversible *in vitro* [Quastel & Wheatley, 1934; Jowett, 1938]. McClure *et al.* [1939] have demonstrated the existence of anoxia in the central nervous system during the anaesthesia brought about by barbiturates and other narcotics. The fact that glucose oxidation is quantitatively the most important respiratory process in brain *in vivo* [see Quastel, 1939] makes it likely that *in vivo*, as well as *in vitro*, narcotics exert inhibitory effects on glucose oxidation by the brain.

Narcotics do not inhibit all oxidative processes to the same extent. Urethane, for example, at concentrations which inhibit succinic dehydrogenase has no effect on xanthine oxidase [Sen, 1931]. Barbiturates, chloretone and hyoscine, at concentrations which strongly inhibit glucose oxidation in the brain, have little effect on the oxidations by the same tissue of sodium succinate or of *p*-phenylenediamine [Quastel & Wheatley, 1932].

Narcotics include a large variety of structural types, such as hydrocarbons, alcohols, ethers, urethanes, sulphones, amides etc., and it is evident that their common characteristic of inducing narcosis must depend upon certain physicochemical characters which they have in common rather than upon the possession of any special chemical structure. The well-known Overton-Meyer theory suggests one important factor in the determination of narcotic activity, i.e. the relative solubilities in lipoid and non-lipoid substances. It is evident, however, that if the nonpolar-polar character of a substance may determine, or partly determine, its entry into a cell, it gives no obvious clue to the mechanism of action of the narcotic once it is within the cell.

It is well known that many enzymic reactions taking place in cell-free extracts are inhibited by narcotics if these are present at sufficiently high concentrations. The mechanism of this inhibition is usually held to be due to a non-specific adsorption on the enzyme surface. So far as respiratory processes are concerned, the relative lack of inhibitory action of narcotics on the indophenol (or cytochrome) oxidase has made it appear that the dehydrogenases are the most sensitive structures to narcotic inhibition.

A study [Jowett & Quastel, 1937; Jowett, 1938] of the inhibitions of respiration in intact brain tissue secured by narcotics has revealed the following facts.

(1) The inhibitions for a large class of narcotics vary little with time and are reversible, i.e. if the narcotic is washed away from the tissue the latter tends to respire again at the normal rate. Such a class includes urethane, chloral, chloretone, barbiturates such as luminal and evipan, avertin (tribromoethyl alcohol) and Mg ions. The narcotic appears to reach a mass action equilibrium with some component of the respiratory system, the inhibition being given by a simple mass action equation.

(2) The steady reversible inhibitions depend on cation activity and particularly on the potassium ion concentration of the medium bathing the brain tissue. If the potassium concentration becomes low, say one-third of that present in blood, the inhibitions increase with time, often considerably. The effect of higher potassium concentration is to stabilize the inhibition. This phenomenon would be explained if, in the presence of low concentrations of potassium ions, a factor important in influencing narcotic inhibitions diffuses from the brain slices into the outer medium.

(3) The inhibition of respiration depends on the concentration of narcotic and not upon the concentration of the substrate which is oxidized.

When, however, anaerobic oxidations accomplished by brain tissue are considered, e.g. the oxidation of lactic acid by methylene blue, it is found that the inhibition by a narcotic does depend on the substrate concentration [Davies & Quastel, 1932]. There exists an equilibrium between lactic acid and the narcotic in presence of the lactic dehydrogenase of brain, to which the mass action law is applicable. There is thus an apparent discrepancy between the two results—an equilibrium between the substrate and the narcotic under anaerobic conditions, and a lack of such equilibrium under aerobic conditions. Yet under suitable ionic conditions an equilibrium does exist under aerobic conditions between the narcotic and some component of the respiratory system.

A clue to the solution of this anomaly may be found in the fact that the low concentrations of narcotics which are highly inhibitory aerobically have but little inhibitory effect anaerobically. Thus chloretone is ten times less effective in securing an inhibition of lactic acid oxidation in presence of brain anaerobically than under aerobic conditions.

A possible explanation is that the narcotic, at the low concentrations effective under aerobic conditions, exerts its main effect on a part of the respiratory system which is inert or functionless under anaerobic conditions. That such a part of the respiratory system has the properties of an enzyme structure is evident from the fact that it is affected by surface-active narcotics of so many different chemical types. It must, however, be an enzyme with a much higher affinity for narcotics than the most sensitive enzymes—the dehydrogenases—with which we are so far familiar.

If this view is true the inhibitions of respiration of intact brain tissue obtained by low concentrations of narcotics under aerobic conditions are not due to competition of the narcotics with the substrates (e.g. lactic or pyruvic acid) for their dehydrogenases, but to the affinity of the narcotics to a special enzyme structure which plays a highly important part in the chain of reactions composing the complete aerobic respiratory process of the cell.

It is the purpose of this communication to throw some light on the site of the enzyme system which is highly sensitive to surface-active narcotics.

Experimental technique

Measurement of oxygen uptake

The Warburg respiration apparatus has been used exclusively in this investigation. The volume of fluid in the reaction vessel is always 3 ml., the *p*H being kept at 7·4 by means of sodium phosphate buffer. In anaerobic experiments sodium bicarbonate solution (0·025 *M*) is used in an atmosphere of 95 % N_2 + 5 % CO_2. The ferricyanide technique of Quastel & Wheatley [1938] has been used in all the anaerobic work and the reader is referred to their paper for full experimental details. Experiments are usually run for 1 hr. at 37°.

Biological material

The biological material used in this work consists of thin slices of brain cortex (rat or guinea-pig), or of minced brain tissue, or of various preparations of tissue enzyme systems. The latter have been made by methods which are already fully described in the literature.

Narcotic investigated

Chloretone ($CCl_3.C(CH_3)_2OH$) is the narcotic extensively used in this investigation. This narcotic has been used because its solubility is such that it can be used conveniently in the Warburg apparatus, 0·2 ml. of a 0·5 % aqueous solution usually being placed in the side tube of the reaction vessel. This amount of chloretone on being tipped into the reaction vessel, after temperature equilibration, usually secures a large inhibition of the rate of O_2 uptake of the tissue (intact brain slices or minced brain). This drug is also used because it brings about, under suitable conditions, steady reversible inhibitions. It is also neutral in reaction, has no buffering value and is suitable for the anaerobic as well as the aerobic work. It appears to be quite stable, and dilute solutions can be kept at 0° for long periods without deterioration. It has the properties of a surface-active narcotic and has already been much investigated in this laboratory [see Jowett & Quastel, 1937; Jowett, 1938].

Effect of chloretone on brain respiration in presence of glucose

A few typical examples of the large inhibitory effects of chloretone on the respiration of brain cortex slices or of minced brain in a glucose-Locke medium are shown in Table 1. Under the conditions cited 0·033 % chloretone brings

Table 1. *Effect of chloretone on brain respiration in presence of glucose*

Exp. 1. Brain cortex slices in sodium phosphate (0·03 *M*), *p*H 7·4-Locke medium (K^+ = 0·004 *M*; Ca^{++} = 0·002 *M*) containing glucose (0·016 *M*); oxygen; 37°.

Animal	Chloretone concentration %	Q_{O_2}	Inhibition %
Rat	Nil	11·8	—
Rat	0·033	4·0	66
Guinea-pig	Nil	11·0	—
Guinea-pig	0·016	5·3	52

Exp. 2. Minced rat brain washed once with Locke solution and suspended in Locke solution (1 g./10 ml.); 1 ml. brain suspension in phosphate (0·03 *M*) *p*H 7·4-Locke-glucose (0·016 *M*) medium; air; 37°.

	μl. O_2 uptake in 1 hr.	Inhibition %
No chloretone present	181	—
With chloretone 0·033 %	120	33·7
With chloretone 0·066 %	59	67

about an inhibition of 66 % in the respiration of rat brain cortex slices and an inhibition of 33 % in the respiration of minced rat brain tissue. The respiration of brain cortex slices in a glucose medium is usually more sensitive to narcotics than that of minced tissue, a fact already noted by Jowett [1938] using ethyl-urethane.

Effect of chloretone on anaerobic glycolysis in presence of brain slices

If the action of chloretone on glucose oxidation in brain cortex slices is due to an inhibitory effect upon dehydrogenases involved in the aerobic breakdown of glucose, it may be expected that this effect will be exerted also on the dehydrogenases involved in the anaerobic breakdown of glucose. The mechanism of anaerobic breakdown of glucose in brain is not yet clear, but if the reactions involved bear some resemblance to those taking part in the anaerobic breakdown of glucose in muscle or yeast, an interplay of dehydrogenase systems will be involved. Experiments (see Table 2) show that 0·033 % chloretone exerts no inhibitory effect whatever on the anaerobic breakdown of glucose to lactic acid in presence of rat brain cortex slices. The result shows that if a narcotic-sensitive dehydrogenase system plays an important role in the aerobic breakdown of glucose, it is either absent from, or is without influence in, the reactions involved in the anaerobic breakdown of glucose by brain.

Table 2. *Effect of chloretone on anaerobic glycolysis by brain*

Rat brain cortex slices in sodium bicarbonate (0·025 *M*)-Locke solution containing 0·025 *M* glucose and 0·003 *M* sodium pyruvate; 95 % N_2 + 5 % CO_2; 37°.

	$Q_M^{N_2}$		
Medium without added chloretone	15·2	19·8	16·5
Medium with chloretone 0·033 %	15·1	—	—
Medium with chloretone 0·05 %	—	17·8	16·4

Anaerobic oxidation of glucose by brain tissue

A more direct approach to the problem as to whether a narcotic exerts large inhibitory effects on dehydrogenases involved in glucose breakdown was made in the following manner.

In the ferricyanide technique of Quastel & Wheatley [1938] use is made of the fact that in the reduction of 1 mol. ferricyanide 1 mol. acid is formed and in bicarbonate media this gives rise to 1 mol. CO_2 which is estimated manometrically. The relevant equations are:

$$H + Fe(CN)_6''' \rightarrow H^+ + Fe(CN)_6''''$$
$$H^+ + HCO_3' \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O.$$

The technique has been used for the study of cozymase-linked systems (since ferricyanide readily oxidizes reduced cozymase [Haas, 1937]), as well as for the study of other dehydrogenase systems (e.g. those involving succinic acid or choline) which do not depend for their activity on cozymase. The technique can be used in the investigations of anaerobic oxidations brought about by intact tissue slices as well as by minced tissue or by tissue extracts. Full experimental details may be found in the paper quoted.

The addition of ferricyanide solution containing a little bicarbonate (1 ml. 0·16 *M* $NaHCO_3$ to 5 ml. 10 % $Na_3Fe(CN)_6$) to minced brain tissue in a bicarbonate-glucose medium in an atmosphere of 95 % N_2 + 5 % CO_2 results in

but little evolution of CO_2 above that obtained, by glycolysis, in the absence of ferricyanide. The addition of pyocyanine to the medium, followed by that of ferricyanide, brings about a very large increase in the velocity of CO_2 output. Pyocyanine is reduced in the presence of brain tissue by glucose, or by a breakdown product of glucose, and the reduced pyocyanine is oxidized directly by ferricyanide. Pyocyanine in this way acts as a catalyst in the anaerobic oxidation of glucose (or of a breakdown product of glucose) by ferricyanide. A typical result with minced rat brain tissue is shown in Table 3. In this case a CO_2 output of 144·5 μl. in the presence of glucose and ferricyanide is increased to 520·2 μl. by the addition of pyocyanine.

The same phenomenon is observed with brain cortex slices. A typical result is given in Table 4. The addition of pyocyanine increases the $Q^{N_2}_{CO_2}$ (μl. CO_2 produced per hr. per mg. dry weight of tissue) from 17·8 to 37·8.

Table 3. *Anaerobic oxidation of glucose, lactate and pyruvate by minced brain*

Minced brain tissue washed with saline and suspended in saline; 1 ml. suspension in bicarbonate (0·025 *M*)-saline; glucose, 0·016 *M*; sodium lactate, 0·02 *M*; sodium pyruvate, 0·015 *M*; pyocyanine chloride, 1·6 mg. per 100 ml. 0·2 ml. 8·3 % sodium ferricyanide solution added where indicated; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output		
Substrates etc. added	Rat 0·1 g. wet wt. tissue (1 hr.)	Rat 0·15 g. wet wt. tissue (1 hr.)	Guinea-pig 0·2 g. wet wt. tissue (2 hr.)
Ferricyanide	45·0	86·8	51·5
Pyocyanine + ferricyanide	175·5*	113·3	61·7
Glucose	105·0	—	—
Glucose + ferricyanide	144·5	—	—
Lactate + ferricyanide	—	107·2	—
Pyruvate + ferricyanide	—	—	68·4
Glucose + pyocyanine + ferricyanide	520·2*	—	—
Lactate + pyocyanine + ferricyanide	—	536·4	—
Pyruvate + pyocyanine + ferricyanide	—	—	248·0

* Pyocyanine chloride = 3·2 mg. per 100 ml.

Table 4. *Effect of chloretone on anaerobic oxidation of glucose by brain*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % sodium ferricyanide solution added where indicated; pyocyanine chloride, 3·2 mg. per 100 ml.; glucose, 0·01 *M*; 95 % N_2 + 5 % CO_2; 37°.

	$Q^{N_2}_{CO_2}$	
Glucose + ferricyanide	17·8	
Pyocyanine + ferricyanide	8·5	
Glucose + pyocyanine	12·1	13·7
Glucose + pyocyanine + ferricyanide	37·8	31·2
Glucose + pyocyanine + ferricyanide + chloretone (0·083 %)	29·5	26·8
Inhibition by chloretone	21·9 %	14·1 %

Effect of chloretone on the anaerobic oxidation of glucose by brain cortex slices

The presence of small concentrations of chloretone which inhibit vigorously the aerobic oxidation of glucose has no effect on the anaerobic oxidation of glucose by brain tissue in the presence of pyocyanine and ferricyanide. Even so high a concentration as 0·083 % chloretone, which inhibits the O_2 uptake of rat brain cortex slices in a glucose medium by over 80 %, only inhibits the anaerobic oxidation of glucose by pyocyanine and ferricyanide by about 20 %. Typical

results are noted in Table 4. This result indicates the relative insensitivity to chloretone of the dehydrogenase system responsible for the reduction of pyocyanine by brain tissue in presence of glucose.

It is possible that the substance which brings about the reduction of pyocyanine in the experiment just quoted is lactic acid, produced from glucose by glycolysis, and that the dehydrogenase system involved is that mainly concerned with the oxidation of lactic acid.

The effects of chloretone on the aerobic and anaerobic oxidations of lactic acid in the presence of brain will, therefore, now be considered.

Effects of chloretone on the aerobic and anaerobic oxidations of lactic acid

As with the oxidation of glucose, narcotics exercise a large inhibition on the aerobic oxidation of lactic acid in the presence of brain. A few typical results, obtained both with brain cortex slices and minced brain tissue, are shown in Table 5. The inhibition by chloretone is of the same order of magnitude as with glucose oxidation.

Table 5

I. Guinea-pig brain cortex slices in Na phosphate (0·03 *M*) *p*H 7·4-Locke solution; sodium lactate, 0·02 *M*; O_2; 37°.

	Q_{O_2}	
Lactate	13·4	16·5
Lactate + chloretone 0·016 %	6·9	7·5
Inhibition by chloretone	49 %	54 %

II. Minced washed rat brain suspension (1 g. to 10 ml.) in Na phosphate (0·03 *M*) *p*H 7·4-saline; 1 ml. suspension taken; sodium lactate, 0·01 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.	
Lactate	249·1	194
Lactate + chloretone 0·083 %	60·0	—
Lactate + chloretone 0·033 %	—	129

Pyocyanine greatly accelerates the anaerobic oxidation by ferricyanide of lactic acid in presence of brain tissue, as may be seen by a glance at Table 3. Chloretone, however, even at the concentration of 0·083 % exerts no inhibitory effect on the anaerobic oxidation of lactic acid by pyocyanine and ferricyanide in presence of rat brain cortex slices (see Table 6).

Table 6. *Effect of chloretone on anaerobic oxidation of lactate*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % Na ferricyanide solution added; sodium lactate, 0·016 *M*; pyocyanine chloride, 3·2 mg. per 100 ml.; 95 % N_2 + 5 % CO_2; 37°.

	$Q^{N_2}_{CO_2}$
Pyocyanine + ferricyanide + lactate	20·8
Pyocyanine + ferricyanide + lactate + chloretone 0·083 %	19·6

If, therefore, the lactic dehydrogenase system responsible for the reduction of pyocyanine by lactic acid is identical with that which brings about the activation of lactic acid under aerobic conditions, the latter cannot be the narcotic-sensitive component of the aerobic respiratory mechanism.

Effect of chloretone on the aerobic and anaerobic oxidations of pyruvic acid by brain tissue

Pyruvic acid oxidation by brain, either in the form of cortex slices or of a mince, is as sensitive to the action of chloretone as the oxidation of glucose or of lactic acid. Typical results are shown in Table 7, in which it is also shown that the addition of pyocyanine under aerobic conditions has no appreciable effect upon the chloretone inhibition.

Table 7

I. Rat brain cortex slices in Na phosphate (0·03 *M*, *p*H 7·4)-Locke-Na pyruvate (0·016 *M*) medium; O_2; 37°.

	Q_{O_2}
No chloretone present	10·1
With chloretone 0·033 %	3·5
Inhibition by chloretone	66 %

II. Minced guinea-pig brain washed thrice with saline and suspended in saline (1 g. to 5 ml.); 1 ml. suspension in Na phosphate (0·03 *M*, *p*H 7·4)-saline; Na pyruvate, 0·015 *M*; pyocyanine chloride, 1·6 mg. per 100 ml.; air; 37°.

Substrates etc. added	μl. O_2 uptake in 1 hr.
Nil	30·1
Pyocyanine	36·6
Na pyruvate	105·3
Na pyruvate + pyocyanine	99·1
Na pyruvate + chloretone 0·083 %	6·9 (inhibition = 93 %)
Na pyruvate + pyocyanine + chloretone 0·083 %	10·8 (inhibition = 89 %)

Anaerobically, pyruvic acid is but feebly attacked by ferricyanide in presence of brain cortex slices. The addition of pyocyanine greatly accelerates the rate of oxidation by ferricyanide, a $Q_{CO_2}^{N_2}$ of 6·6 in the absence of pyocyanine being increased to 17·9 in its presence. The addition of a relatively high concentration of chloretone has but little inhibitory effect upon the anaerobic oxidation of pyruvic acid by rat brain cortex slices. A representative result is shown in Table 8.

Table 8. *Effect of chloretone on anaerobic oxidation of pyruvate*

Rat brain cortex slices in bicarbonate (0·025 *M*)-Locke medium; 0·2 ml. 8·3 % Na ferricyanide added where indicated; pyocyanine chloride, 3·2 mg. per 100 ml.; Na pyruvate, 0·016 *M*; 95 % N_2 + 5 % CO_2; 37°.

	$Q_{CO_2}^{N_2}$
Ferricyanide	4·1
Pyocyanine + ferricyanide	8·5
Pyruvate + ferricyanide	6·6
Pyruvate + pyocyanine + ferricyanide	17·9
Pyruvate + pyocyanine + ferricyanide + chloretone (0·083 %)	13·1
Inhibition by chloretone	26·8 %

It follows from these results that if a pyruvic dehydrogenase is common to both the aerobic and anaerobic oxidations of pyruvic acid in brain, this enzyme cannot be regarded as the narcotic-sensitive component of the aerobic system.

Effect of chloretone on the anaerobic oxidation of lactic acid in presence of crushed yeast

Lactic acid is vigorously oxidized in presence of a suspension of dialysed crushed baker's yeast by ferricyanide under anaerobic conditions in the absence of any added carrier such as pyocyanine. The crushing of the yeast was accom-

plished in a wet-crushing mill [Booth & Green, 1938] and the crushed yeast was dialysed against distilled water overnight in the ice chest.

The addition of potassium ferricyanide solution to a suspension of the crushed yeast in a bicarbonate medium results in an evolution of CO_2, due to the oxidation of substances still present in the suspension. The further addition of sodium lactate results in a large acceleration of the rate of CO_2 output. It will be seen from Table 9 that an evolution of 210 μl. CO_2 in 10 min. was increased, by the presence of lactate, to 683·5 μl. CO_2 in the same time.

Oxidation of lactic acid, in presence of crushed yeast, by ferricyanide occurs also on addition of sodium cyanide. The rate is not materially affected by the addition of a cozymase preparation (a slight increase in the rate due to added cozymase is noted in Table 9).

Table 9

Baker's yeast (40 g. yeast and 20 ml. water) crushed in mill and dialysed against distilled water; 1 ml. suspension in bicarbonate (0·025 *M*)-saline; 0·2 ml. 8·3 % K ferricyanide solution added; 95 % N_2 + 5 % CO_2; 37°.

	Substances added	μl. CO_2 output in 10 min.
I.	Ferricyanide	210·0
	Ferricyanide + chloretone (0·083 %)	200·1
	Na lactate (0·015 *M*) + ferricyanide	683·5
	Na lactate (0·015 *M*) + ferricyanide + chloretone (0·083 %)	604·9
		μl. CO_2 output in 30 min.
II.	NaCN (0·03 *M*) + ferricyanide	86·0
	Na lactate (0·01 *M*) + NaCN (0·03 *M*) + ferricyanide	638·3
	Na lactate (0·01 *M*) + NaCN (0·03 *M*) + 1 mg. cozymase + ferricyanide	705·6

The addition of chloretone (0·083 %) depresses only slightly the rate of oxidation of lactic acid by ferricyanide in presence of yeast lactic dehydrogenase (Table 9).

It is evident, therefore, that yeast lactic dehydrogenase, like that of brain, is not highly sensitive to the narcotic.

Effect of chloretone on muscle dehydrogenase

A preparation of a dialysed extract of rabbit skeletal muscle was used as a source of muscle dehydrogenase. It was made according to the method of Green *et al.* [1937]. As shown by Quastel & Wheatley [1938], this extract contains dehydrogenases which, in the presence of cozymase and the appropriate substrates, rapidly reduce ferricyanide under anaerobic conditions.

Sodium hexosediphosphate and sodium α-glycerophosphate are examples of substrates which are oxidized rapidly by ferricyanide in the presence of rabbit skeletal muscle extract containing cozymase.

Chloretone (0·083 %) does not inhibit the anaerobic oxidation of hexosediphosphate by this system.

The oxidation of α-glycerophosphate is inhibited by chloretone (0·033 %) to the extent of 10 %, the inhibition being increased to 30 % when this concentration of chloretone is doubled. These facts are noted in Table 10.

It seems, therefore, that although the α-glycerophosphate dehydrogenase system of skeletal muscle shows some sensitivity to the narcotic, it is not nearly as sensitive as the aerobic respiratory systems of intact brain tissue in glucose media.

Table 10

Rabbit skeletal muscle extract dialysed; 1 ml. extract in 0·025 *M* bicarbonate-saline; 0·2 ml. 8·3 % K ferricyanide added where indicated; 1 mg. cozymase preparation added; Na hexosediphosphate, 0·23 %; Na α-glycerophosphate, 0·02 *M*; 95 % N_2 + 5 % CO_2; 37°.

		μl. CO_2 output in 15 min.
I.	Na hexosediphosphate + cozymase	65·1
	Na hexosediphosphate + cozymase + ferricyanide	409·2
	Na hexosediphosphate + cozymase + ferricyanide + chloretone (0·083 %)	459·0
		μl. CO_2 output in 1 hr.
II.	Na α-glycerophosphate + cozymase + ferricyanide	466·4
	Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·016 %)	436·3
	Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·033 %)	416·3
	Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·05 %)	411·0
	Na α-glycerophosphate + cozymase + ferricyanide + chloretone (0·066 %)	323·4

Effects of chloretone on an oxidizing system composed of rabbit skeletal muscle extract and crushed baker's yeast

The oxygen uptake obtained when sodium α-glycerophosphate or sodium hexosediphosphate is incubated at 37° with dialysed crushed baker's yeast is much increased when dialysed rabbit skeletal muscle extract is added to the mixture. This takes place although the incubation of sodium α-glycerophosphate or hexosediphosphate with the muscle extract alone leads to no consumption of oxygen. The increased oxygen uptake is doubtless due to the fact that the muscle extract supplies increased amounts of dehydrogenases, which in presence of cozymase and the cytochrome system provided by the crushed yeast, lead to oxidation of the α-glycerophosphate and hexosediphosphate. Typical results are shown in Table 11 (A and B) and Table 12 (A).

Table 11. *Rabbit skeletal muscle extract and crushed baker's yeast (20 %)*

A. 1 ml. dialysed muscle extract, 0·5 ml. crushed yeast in Na phosphate (0·03 *M*), *p*H 7·4-saline; Na α-glycerophosphate, 0·02 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.
Muscle extract + α-glycerophosphate	0·0
Crushed yeast + α-glycerophosphate	89·3
Muscle extract + crushed yeast + α-glycerophosphate	198·5

B. 1·5 ml. dialysed muscle extract and 0·2 ml. dialysed crushed yeast in Na phosphate, *p*H 7·4-saline; Na α-glycerophosphate, 0·02 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.
Crushed yeast + α-glycerophosphate	66·4
Crushed yeast + α-glycerophosphate + muscle extract	188·3
Crushed yeast + α-glycerophosphate + muscle extract + chloretone (0·05 %)	89·3
Inhibition by chloretone	51·7 %

C. 1·5 ml. dialysed muscle extract and 0·2 ml. dialysed crushed yeast in bicarbonate (0·025 *M*)-saline; Na α-glycerophosphate, 0·02 *M*; 0·2 ml. 8·3 % K ferricyanide; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Ferricyanide + muscle extract + crushed yeast + α-glycerophosphate	343·8
Ferricyanide + muscle extract + crushed yeast + α-glycerophosphate + chloretone (0·05 %)	260·4
Inhibition by chloretone	24·3 %

Table 12. *Rabbit skeletal muscle extract and crushed baker's yeast*

A. 1·5 ml. dialysed rabbit muscle extract and 0·2 ml. dialysed crushed baker's yeast in Na phosphate (0·03 *M*), *p*H 7·4-saline; Na hexosediphosphate, 0·005 *M*; air; 37°.

	μl. O_2 uptake in 1 hr.
Crushed yeast + hexosediphosphate	37·3
Crushed yeast + hexosediphosphate + muscle extract	129·9
Crushed yeast + hexosediphosphate + muscle extract + chloretone (0·05 %)	60·4
Inhibition by chloretone	53·4 %

B. 1·5 ml. dialysed rabbit muscle extract and 0·2 ml. dialysed crushed baker's yeast in Na bicarbonate (0·025 *M*)-saline; Na hexosediphosphate, 0·005 *M*; 0·2 ml. 8·3 % K ferricyanide; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide	628·9
Crushed yeast + muscle extract + hexosediphosphate + ferricyanide + chloretone (0·05 %)	620·1
Inhibition by chloretone	1·2 %

The addition of chloretone (0·05 %) to the system—skeletal muscle extract and dialysed crushed yeast—results in over 50 % inhibition of the oxygen uptake obtained in the presence of either sodium α-glycerophosphate (Table 11B) or of sodium hexosediphosphate (Table 12).

The system of muscle extract and crushed yeast can be investigated anaerobically using the ferricyanide technique. Under such circumstances the cytochrome system presumably plays no part, and the reduction of ferricyanide will be accomplished through the activities of the combined dehydrogenases of the muscle extract and crushed yeast, the cozymase necessary for the reduction being supplied largely by the yeast. Investigation of this system shows that the addition of chloretone (0·05 %) brings about little or no inhibition of the anaerobic oxidation of hexosediphosphate (Table 12B) and 24 % inhibition of the rate of anaerobic oxidation of α-glycerophosphate (Table 11C).

These results show that whilst the oxygen uptake of a respiratory system—muscle extract, crushed yeast and substrate—is considerably inhibited by a narcotic, the amount of inhibition by the same concentration of narcotic is greatly decreased when the system is examined anaerobically. Since the dehydrogenases therefore cannot be the narcotic-sensitive component of the respiratory system, it follows that the narcotic must act vigorously on some component occupying an intermediate position between the dehydrogenase and the cytochrome systems.

Effect of chloretone on an oxidizing system composed of rabbit skeletal muscle extract, cozymase and cytochrome oxidase

Substantially the same results as those just recorded are obtained if crushed yeast be replaced by a mixture of cozymase and cytochrome oxidase, prepared from pig heart according to the method of Keilin & Hartree [1938]. A typical result given in Table 13A shows that oxygen consumption takes place only in the complete system—cytochrome oxidase, muscle extract, sodium α-glycerophosphate and cozymase. The cytochrome and flavoprotein necessary for the system are present in the cytochrome oxidase preparation.

Addition of chloretone (0·083 %) to this system brings about over 80 % inhibition of the rate of oxygen uptake (Table 13B). Thus a relatively narcotic-insensitive system—muscle extract, α-glycerophosphate, cozymase—is made narcotic-sensitive by the addition to it of a cytochrome oxidase preparation.

Table 13. *Rabbit muscle extract and pig heart muscle (cytochrome oxidase)*

1 ml. dialysed rabbit muscle extract and 0·5 ml. cytochrome oxidase preparation in Na phosphate (0·03 *M*) *p*H 7·4-saline; 1 mg. cozymase preparation; Na α-glycerophosphate, 0·02 *M*; air; 37°.

		μl. O_2 uptake in 1 hr.
A.	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate	0·0
	Cytochrome oxidase preparation + cozymase + α-glycerophosphate	0·0
	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase	96·1
B.	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase	136·0
	Cytochrome oxidase preparation + muscle extract + α-glycerophosphate + cozymase + chloretone (0·083 %)	16·7
	Inhibition by chloretone	87 %

Effect of chloretone on an oxidizing system composed of glucose dehydrogenase, cozymase and cytochrome oxidase

A water-soluble preparation of glucose dehydrogenase was made by acetone treatment of pig liver according to the method of Harrison [1931]. This preparation, as shown by Hawthorne & Harrison [1939], in presence of cozymase, cytochrome, cytochrome oxidase and glucose (probably with the co-operation of a flavoprotein) takes up oxygen, the glucose being oxidized to gluconic acid. Only the complete system takes up oxygen as shown by the results given in Table 14. Cytochrome was, in fact, not added in the experiment recorded in Table 14, as the cytochrome oxidase preparation contained sufficient quantities of it.

Table 14. *Glucose dehydrogenase (acetone pig liver) preparation and cytochrome oxidase (pig heart) preparation*

I. 1 ml. glucose dehydrogenase preparation (0·25 g. in 8 ml. saline), 0·5 ml. cytochrome oxidase preparation, 1 mg. cozymase preparation in Na phosphate (0·03 *M*), *p*H 7·4-saline; glucose, 0·016 *M*; air; 37°.

		μl. O_2 in 1 hr.
	Glucose dehydrogenase + cozymase + glucose	0·0
	Glucose dehydrogenase + cytochrome oxidase + glucose	6·0
	Glucose dehydrogenase + cytochrome oxidase + cozymase	5·0
	Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose	80·6
II.	Glucose dehydrogenase (1 ml. of solution of 0·5 g. in 8 ml. saline) + cytochrome oxidase + cozymase + glucose (0·1 *M*)	341·2
	Glucose dehydrogenase + cytochrome oxidase + cozymase + glucose + chloretone (0·083 %)	8·4
	Inhibition by chloretone	97 %

The complete system—glucose dehydrogenase, cytochrome oxidase, cozymase and glucose—is highly sensitive to chloretone. This narcotic at a concentration of 0·083 % brings about over 90 % inhibition of the rate of oxygen uptake (Table 14).

The system may be investigated anaerobically by the ferricyanide technique. Results in Table 15 (I) show that the addition of cozymase greatly increases the rate of CO_2 output obtained by incubating anaerobically the glucose dehydrogenase preparation with glucose and ferricyanide. The system is thus a typical cozymase-linked dehydrogenase system.

The addition of chloretone at a concentration of 0·083 % secures no inhibition within experimental error of the rate of anaerobic oxidation of glucose by ferricyanide in presence of glucose dehydrogenase and cozymase (Table 15).

Table 15. *Glucose dehydrogenase (acetone pig liver) preparation*

I. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0·025 *M* Na bicarbonate-saline; glucose, 0·016 *M*; 0·2 ml. 8·3 % K ferricyanide solution added; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 1 hr.
Glucose dehydrogenase	88·9
Glucose dehydrogenase + cozymase	96·0
Glucose dehydrogenase + glucose	68·5
Glucose dehydrogenase + cozymase + glucose	233·3

II. 1 ml. glucose dehydrogenase preparation; 1 mg. cozymase preparation; 0·025 *M* Na bicarbonate-saline; glucose, 0·1 *M*; 0·2 ml. 8·3 % K ferricyanide added; 95 % N_2 + 5 % CO_2; 37°.

	μl. CO_2 output in 30 min.
Glucose dehydrogenase + cozymase	75·4
Glucose dehydrogenase + cozymase + chloretone (0·083 %)	75·6
Glucose dehydrogenase + cozymase + glucose	335·8
Glucose dehydrogenase + cozymase + glucose + chloretone (0·083 %)	329·7
Inhibition by chloretone	2 %

The conclusion must be drawn that, as with the α-glycerophosphate system of muscle, the glucose dehydrogenase of liver is relatively narcotic-insensitive, and that the glucose-oxidizing system becomes highly narcotic-sensitive on the addition to it of a cytochrome oxidase preparation.

Chloretone and cytochrome oxidase

It is well known that cytochrome oxidase is relatively insensitive to narcotics. Quastel & Wheatley [1932] for example showed that chloretone and barbiturates, at concentrations which greatly inhibited the respiration of brain in glucose media, have little or no effect upon the oxidation of *p*-phenylenediamine or of Na succinate by brain tissue. It must be concluded that the narcotic inhibits neither the mechanism by which oxygen enters the cell nor that by which it is brought into a state of activity.

Chloretone and the aerobic respiratory system

The observations which have been recorded so far may be summarized as follows:

1. Chloretone, at the small concentrations which inhibit the respiration of intact brain tissue, has but little inhibitory effect upon the dehydrogenase systems which may be expected to play a part in respiration. This insensitivity of the known dehydrogenases towards the small but biologically important concentrations of narcotic probably accounts for the fact that the inhibition of respiration by a narcotic is independent of the concentration of the substance burned [Jowett & Quastel, 1937].

2. Chloretone has little or no inhibitory effect on the activity of cytochrome oxidase.

3. A respiratory system composed of a mixture of a dehydrogenase system and a cytochrome oxidase preparation—both of which components are separately relatively narcotic-insensitive—is narcotic-sensitive.

The conclusion to be drawn from these observations is that the component in the complete respiratory system which is highly narcotic-sensitive must be a factor playing an intermediate role between the dehydrogenase and cytochrome

oxidase systems. This factor must be lacking in the respiratory system involving the aerobic oxidation of succinate in animal tissues, since this is relatively narcotic-insensitive.

The narcotic-sensitive factor in the system involving the oxidation of lactic acid by animal tissues must take part in the reactions between reduced cozymase and cytochrome (or one of the cytochromes). A number of substances may be involved in this intermediate section of the respiratory system and these are not yet clearly defined or identified.

The evidence points, however, to the fact that a flavoprotein plays an important role in this part of the respiratory system. A flavoprotein (diaphorase) is known to be reduced by reduced cozymase and there is some evidence that reduced flavoproteins may react with components of the cytochrome system [Theorell, 1936; Dewan & Green, 1938]. Haas *et al.* [1940] have reported the existence of a flavoprotein in yeast which completes the oxidation-reduction chain between hexosemonophosphate and cytochrome *c*.

Flavoprotein, together with the cytochrome components, is present in the cytochrome oxidase preparation used in the experiments of which the results are given in Tables 13 and 14.

An investigation, therefore, was made of the effects of chloretone on the accelerating action of flavoprotein in an oxidizing system.

Chloretone and flavoprotein

Heart muscle flavoprotein was prepared by the method of Straub [1939]. Its catalytic activity in oxidation has been demonstrated by Corran *et al.* [1939]. As shown by these authors the oxygen uptake of a system composed of rabbit skeletal muscle extract, cozymase, sodium lactate, sodium cyanide and methylene blue is much increased by the addition of flavoprotein. A typical result is shown in Table 16 (I). The flavoprotein is reduced by reduced cozymase, and methylene blue is in turn reduced by the reduced flavoprotein. The reduced methylene blue is autoxidizable, and hence a chain of reactions securing the oxidation of lactate is formed. Cyanide is added to the system to fix the pyruvate, which would otherwise inhibit the activity of the lactic dehydrogenase of the muscle extract.

Table 16

Constituents present in Na phosphate (0·02 *M*) *p*H 7·4-saline medium: 0·3 ml. dialysed rabbit skeletal muscle extract, 0·5 ml. flavoprotein preparation, 1 mg. cozymase preparation, Na cyanide (0·083 *M*), Na lactate (0·13 *M*) and methylene blue (0·017 %); air; 37°.

		μl. O_2 uptake in 1 hr.
I.	Muscle extract + cozymase + Na lactate + NaCN + methylene blue	62·5
	Flavoprotein + cozymase + Na lactate + NaCN + methylene blue	44·2
	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	303·2
II.	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue	268·5
	Muscle extract + flavoprotein + cozymase + Na lactate + NaCN + methylene blue + chloretone (0·083 %)	270·5
	% inhibition by chloretone	Nil

Addition of chloretone at a relatively high concentration (0·083 %) has no inhibitory effect on this system (Table 16 (II)).

It follows from this fact that heart flavoprotein is not itself unduly affected by the narcotic. The narcotic appears to affect neither the reduction of flavo-

protein by cozymase nor the reduction of methylene blue by reduced flavoprotein. Yet in a respiratory system consisting of rabbit skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase, sodium lactate and semicarbazide (added to fix the pyruvate) the addition of chloretone (0·083 %) causes 52% inhibition of the velocity of oxygen uptake. This fact is noted in Table 17.

Table 17

Constituents present: 1 ml. dialysed rabbit skeletal muscle extract; 1 ml. flavoprotein preparation, 1 mg. cozymase preparation, 0·2 ml. cytochrome oxidase preparation, semicarbazide 0·03 *M*, Na lactate 0·13 *M* and saline; air; 37°.

	μl. O_2 uptake in 1 hr.
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate	135·1
Muscle extract + cozymase + flavoprotein + cytochrome oxidase + semicarbazide + Na lactate + chloretone (0·083 %)	64·8
Inhibition by chloretone	52 %

Unless, therefore, there is still another enzyme which is highly narcotic-sensitive and which is playing a role between flavoprotein and one or more of the cytochrome components, it must be concluded that either

(*a*) There is present in animal tissues a special narcotic-sensitive flavoprotein which forms an important link between reduced cozymase and the cytochrome system. It should be noted that the cytochrome *c* reductase of Haas *et al.* [1940] is a flavoprotein composed of a specific protein and an alloxazine mononucleotide. It is very unstable compared with other flavoproteins and its activity is destroyed by acetone and dioxane. Conceivably such a flavoprotein may be highly narcotic-sensitive; or

(*b*) Some component of the cytochrome system itself is highly narcotic-sensitive (see e.g. Keilin & Hartree [1939]). This component would obviously play little part in the oxidation of either sodium succinate or of *p*-phenylenediamine in animal tissues.

The oxidation of pyruvate by tissues is greatly inhibited by a narcotic such as chloretone (Table 7) and this system is presumably independent of the action of cozymase. There is some evidence, however [Lipmann, 1939], that a flavoprotein is involved in pyruvate oxidation by bacteria and conceivably a flavoprotein is involved in the oxidation of pyruvate by animal tissues.

The oxidation of *dl*-alanine by rat kidney slices has been shown [Jowett & Quastel, 1937] to be inhibited by small concentrations of a narcotic. Since amino-acid oxidase has been identified as a flavoprotein, it is possible, from what has been said, that the narcotic acts here on the process intermediate between the flavoprotein and a cytochrome component.

With our present state of incomplete knowledge of the components of respiratory systems of animal tissues it would be unwise to speculate further. A solution of the problem of the precise site at which a narcotic acts, in biologically important concentrations, in respiratory systems, depends on a more complete elucidation of the details of these systems. It may be suggested, however, that the property of narcotics of inducing, at low concentrations, strong inhibition of respiratory activity may be used more extensively than hitherto in the investigation of the components of respiratory systems.

What is clear from these results is that, in the inhibition of respiratory activity effected by low concentrations of narcotics, the familiar dehydrogenases, which have been popularly supposed to be the narcotic-sensitive components of

the respiratory processes, are practically unaffected. The narcotic seems to act at a site involving a special flavoprotein or an intermediate between flavoprotein and the cytochrome oxidase system.

Summary

1. The effects of chloretone on the oxidations of glucose, sodium lactate and sodium pyruvate in presence of brain tissue (cortex slices and minced brain), both under aerobic and anaerobic conditions have been investigated. Concentrations of chloretone which effect large inhibitions of the aerobic oxidations have but little effect upon the anaerobic oxidations (using the ferricyanide technique). The dehydrogenases involved in the oxidation of glucose, lactate and pyruvate in presence of brain tissue are (relatively) narcotic-insensitive.

2. Anaerobic glycolysis by brain is not inhibited by relatively high concentrations of chloretone.

3. Anaerobic oxidations of glucose, lactate and pyruvate in the presence of brain tissue and of a carrier such as pyocyanine can be followed by the ferricyanide technique.

4. Anaerobic lactate oxidation by ferricyanide, in presence of crushed yeast, can be followed manometrically in the absence of added carriers. It is but feebly inhibited by relatively high concentrations of chloretone.

5. The addition of crushed yeast to skeletal muscle extract containing sodium α-glycerophosphate produces a narcotic-sensitive respiratory system.

6. The following systems which are but feebly narcotic-sensitive become highly narcotic-sensitive on addition to them of a cytochrome oxidase preparation, viz. dialysed skeletal muscle extract, cozymase and α-glycerophosphate; liver glucose dehydrogenase, cozymase and glucose.

These systems which can be studied manometrically under anaerobic conditions by the ferricyanide technique are not inhibited appreciably by concentrations of chloretone which exercise large inhibitions of the oxygen uptake when pig heart cytochrome oxidase is added.

7. Chloretone at relatively high concentrations does not inhibit the reduction of heart flavoprotein by reduced cozymase, or the reduction of methylene blue by reduced flavoprotein. It exercises a large inhibition of the oxygen uptake of a system composed of skeletal muscle extract, cozymase, flavoprotein, cytochrome oxidase and sodium lactate.

8. The evidence suggests that chloretone at biologically important concentrations affects largely either a special flavoprotein or some component of the tissue respiratory system which plays an intermediate role between flavoprotein and cytochrome oxidase.

Our thanks are due to the Medical Research Council for a grant in aid of the equipment of this laboratory, to the Society for the Protection of Science and Learning for a grant to one of us (M. M.), and to Dr P. J. G. Mann for his help in some of the experimental work described in this paper. We are also grateful to the Ella Sachs Plotz Foundation for a grant for the purchase of animals.

REFERENCES

Booth & Green (1938). *Biochem. J.* **32**, 855.
Corran, Green & Straub (1939). *Biochem. J.* **33**, 793.
Davies & Quastel (1932). *Biochem. J.* **26**, 1672.
Dewan & Green (1938). *Biochem. J.* **32**, 626.
Green, Needham & Dewan (1937). *Biochem. J.* **31**, 2327.
Haas (1937). *Biochem. Z.* **291**, 79.
—— Horecker & Hogness (1940). *J. biol. Chem.* **136**, 747.
Harrison (1931). *Biochem. J.* **25**, 1016.
Hawthorne & Harrison (1939). *Biochem. J.* **33**, 1573.
Jowett (1938). *J. Physiol.* **92**, 322.
—— & Quastel (1937). *Biochem. J.* **31**, 565.
Keilin & Hartree (1938). *Proc. roy. Soc.* B, **125**, 171.
—— —— (1939). *Proc. roy. Soc.* B, **127**, 167.
Lipmann (1939). *Nature, Lond.*, **143**, 436; *Enzymologia* (1937), **4**, 65.
McClure, Hartmann, Schnedorf & Schelling (1939). *Ann. Surg.* **110**, 836.
Quastel (1939). *Physiol. Rev.* **19**, 135.
—— & Wheatley (1932). *Proc. roy. Soc.* B, **112**, 60.
—— —— (1934). *Biochem. J.* **28**, 1521.
—— —— (1938). *Biochem. J.* **32**, 936.
Sen (1931). *Biochem. J.* **25**, 849.
Straub (1939). *Biochem. J.* **33**, 787.
Theorell (1936). *Biochem. Z.* **288**, 317.

54. PROTEIN CATABOLISM AND PROTEIN SPARING UNDER ADRENALINE

By CHARLES REID

From the Department of Physiology, London Hospital Medical College, University of London

(*Received 28 March 1941*)

It has been known for many years that a subcutaneous injection of adrenaline is followed by a rise in O_2 consumption [La Franca, 1909]. Views are not unanimous regarding the nature of the foodstuffs oxidized to furnish the additional calories. To Cori [1931] it appears that under the influence of adrenaline, although the oxidation of carbohydrate is increased a relative suppression of the oxidation of carbohydrate occurs in the intact animal, even in the presence of a carbohydrate plethora, with the result that more than half of the extra calories are supplied by the oxidation of fat [Cori & Cori, 1928]. It is clear, however, from recent work on the isolated heart by Cruickshank & Startup [1934] and by Bogue *et al.* [1935] that the heart under adrenaline uses more O_2 and that the increased O_2 is used preferentially for the combustion of glucose, provided that sufficient glucose is present in the perfusing fluid. Moreover, Paton [1903] and Underhill & Closson [1906] found an increased breakdown of protein under adrenaline when the calorie value of the diet was inadequate.

The fact that adrenaline increases the blood sugar whereas insulin decreases it shows that these hormones have opposing effects as judged by the behaviour of the blood sugar, but the different behaviour of the blood sugar under adrenaline and insulin does not justify the conclusion that the metabolic actions of adrenaline and insulin are necessarily antagonistic as far as the utilization of carbohydrate is concerned.

The administration of carbohydrate lowers protein catabolism in the normal organism. This can readily be shown by giving carbohydrate to a normal fasting animal when the excretion of N promptly decreases. On the other hand, the excretion of N is not lessened by giving carbohydrate to a fasting depancreatized animal. Evidently the protein-sparing action of carbohydrate is linked in some way with the physiological role of insulin. If then adrenaline increases the breakdown of protein in the normal organism but the concomitant administration of carbohydrate prevents this, either largely or entirely, obviously adrenaline does not prevent the protein-sparing action of carbohydrate and so cannot be said to act strictly as an 'anti-insulin' hormone.

Methods

A. *Experiments on anaesthetized cats*

Cats were fasted for 48 hr. and, after anaesthetization with chloralose, were given an intraduodenal injection of about 20 ml. warm water to maintain the flow of urine. After 2 hr. the bladder was emptied and the urine collected for an initial period of 3 hr. Then one of the following was given by slow

infusion into a vein during a period of 5–6 hr., different animals being used for each kind of infusion: (1) adrenaline; (2) glucose; (3) adrenaline + glucose; (4) adrenaline + glucose + insulin; (5) saline. Samples were taken at the beginning and at the end of the 5–6 hr. period for determinations of the blood sugar, liver glycogen, urine sugar and inorganic S. The rate of excretion of inorganic S under the above conditions was taken as indicating the rate of protein catabolism [Reid, 1936] in preference to that of the excretion of N, which is affected considerably by variations in the rate of urine flow. Observations were also made on previously depancreatized cats. The body temperature of the anaesthetized animals was kept constant by means of a rectal thermometer which acted as a thermoregulator for the animal table [Fuchs & Reid, 1937–8].

Results

In intact fasting cats the excretion of inorganic S is increased by infusing adrenaline into a vein [Table 1 (1)].

The increase varies in general with the dose of adrenaline used, viz. 0·02–0·08 mg./kg. body weight/hr. [Table 1 (1)].

Table 1

Nature of infusion			Average % change in hourly rate of excretion of inorganic S during perfusion as compared with control period
Adrenaline mg./kg./hr.	Glucose g./kg./hr.	Insulin unit/kg./hr.	
(1) Intact cats: 6 experiments in each group			
Saline control	—	—	0
0·02	—	—	+22
0·05	—	—	+39
0·08	—	—	+77
0·05	0·30	—	+26
0·08	0·40	—	+32
—	0·26	—	−15
—	0·40	—	−22
0·02	0·40	0·32	−18
0·07	0·60	0·50	+ 8
(2) Previously depancreatized cats: 6 experiments in each group			
0·04	—	—	+53
0·04	0·40	—	+56
0·04	0·40	0·50	+ 2

Thus adrenaline increases the rate of protein catabolism in intact fasting cats.

The administration of glucose along with adrenaline lessens the expected rise in the output of inorganic S [Table 1 (1)].

Glucose, therefore, can spare protein in an animal under the influence of adrenaline.

In previously depancreatized cats adrenaline increases the output of inorganic S [Table 1 (2)] and therefore the rate of protein catabolism, but protein is spared by giving glucose + insulin though not by glucose alone.

It thus seems reasonable to conclude that adrenaline is not strictly an anti-insulin hormone because it does not suppress the ability of the normal organism to use carbohydrate to spare protein. This tentative conclusion is further tested in the following experiments on non-anaesthetized dogs.

B. *Experiments on non-anaesthetized dogs*

Two bitches were subjected during 2 years to the following regime every 2 weeks with or without the administration of adrenaline:

Ordinary feeding for 10 days adjusted so as to keep their weights nearly constant throughout.

Deprivation of food, but not of water, for 2 days.

Catheterization to empty the bladder, with a further 2 days for the collection of urine during this period, and daily feeding by stomach tube with water or glucose solution.

Analysis of the 48 hr. urines for total N, inorganic S and glucose.

When adrenaline was used, it was given subcutaneously in doses of 0·25–1·0 mg. every 3 hr. Auer & Meltzer [1916] have shown that the effects of a subcutaneous dose of adrenaline last for several hours owing to slow absorption. As judged by the behaviour of the pulse rate, body temperature etc. after subcutaneous adrenaline, this certainly appears to be the case. Tissue necrosis may occur with the larger doses used unless injected at more than one site.

Table 2

	Dog *B* (11–12 kg.)		Dog *C* (15–16 kg.)	
Nature of experiment	Total N g./day	Inorganic S g./day	Total N g./day	Inorganic S g./day
Control	2·74	0·104	3·63	0·126
Adrenaline, 0·5 mg./3 hr.	3·29	0·132	4·42	0·147
Adrenaline, 0·5 mg./3 hr. +50 g. glucose daily	2·51	0·097	3·66	0·135
Control	2·68	0·092	3·60	0·118
Glucose, 50 g. daily	2·01	0·071	3·13	0·100
Adrenaline, 0·75 mg./3 hr. +50 g. glucose daily	2·87	0·107	3·80	0·129
Glucose, 80 g. daily	1·90	0·065	2·61	0·077
Adrenaline, 0·75 mg./3 hr.	3·40	0·121	4·08	0·123
Control	2·50	0·080	3·43	0·108

Results

Table 2 gives the data obtained from one of the 4 anoestrous periods in each bitch. Results from periods complicated by oestrus have been excluded. It is clear that, when adrenaline is given by subcutaneous injection to fasting dogs, the urinary output of total N and inorganic S rises, indicating that under these conditions protein catabolism increases. When glucose is given by stomach tube while the animals are under the influence of adrenaline, the urinary output of total N and inorganic S is significantly lessened, so that clearly protein can be spared by carbohydrate even during the acceleration of metabolic processes by adrenaline. The urine was free from sugar except on two occasions when small losses of less than 3 g. were found. The normal fasting dog likewise can use carbohydrate to spare protein (Table 2). Therefore the protein-sparing action of carbohydrate, which appears to be linked with the physiological role of insulin, is not prevented by adrenaline. The latter, accordingly, cannot be regarded strictly as an 'anti-insulin' despite the opposing effects of adrenaline and insulin on the blood sugar.

SUMMARY

Adrenaline has been shown to increase the catabolism of protein, but protein can still be spared by giving carbohydrate, even when the metabolic rate has been raised by adrenaline. The protein-sparing action of carbohydrate appears

to be linked with the physiological role of insulin, since it is not shown under ordinary conditions by the depancreatized animal without insulin. Since the protein-sparing action of carbohydrate still functions when adrenaline is given, adrenaline and insulin do not have strictly antagonistic physiological roles despite their opposing actions on the blood sugar.

REFERENCES

Auer & Meltzer (1916). *Proc. Soc. exp. Biol. N.Y.* **14**, 54.
Bogue, Evans, Grande & Hsu (1935). *Quart. J. exp. Physiol.* **25**, 213.
Cori (1931). *Physiol. Rev.* **11**, 143.
—— & Cori (1928). *J. biol. Chem.* **79**, 343.
Cruickshank & Startup (1934). *J. Physiol.* **80**, 179.
Fuchs & Reid (1937–8). *J. Physiol.* **91**, 10*P*.
La Franca (1909). *Z. exp. Path. Ther.* **6**, 1.
Paton (1903). *J. Physiol.* **29**, 286.
Reid (1936). *J. Physiol.* **87**, 113, 121.
Underhill & Closson (1906). *Amer. J. Physiol.* **17**, 42.

55. A SIMPLIFIED ESTIMATION OF LACTATE IN MUSCLE

By R. E. JOHNSON and A. N. BARGER

From the Fatigue Laboratory, Harvard University, Boston, Massachusetts, U.S.A.

(*Received 10 March 1941*)

Edwards [1938], modifying the method of Friedemann *et al.* [1927], improved the apparatus and simplified the method for estimating lactate in normal human blood. Treatment of blood extracts with copper sulphate and copper hydroxide, preliminary boiling or aeration of extracts and cleaning out the apparatus or adding fresh reagents between runs were all found to be unnecessary, since none of these procedures had any significant effect either on the estimation of preformed lactate or on the recovery of added lactate. We have applied these modifications to deproteinized extracts of muscles, and have also used a simple apparatus for preparing such extracts. The present method can be used with any apparatus suitable for the method of Friedemann *et al.* [1927].

Materials

These are prepared according to Friedemann *et al.* [1927] except as noted below. Instead of sodium bisulphite, a 5 % aqueous solution of sodium metabisulphite is used. This gives slightly better recoveries, especially in warm weather. Excess bisulphite in the titration is removed with $N/3$ iodine in KI. Technical grades of iodine and KI are satisfactory and cost about one-half as much as CP grades. Hydrated disodium phosphate (USP powdered) is used for the adjustment of alkalinity in estimating bound bisulphite. If the product as bought does not contain enough water of crystallization to lower the temperature of water as it dissolves, it is dissolved in an equal weight of hot water and allowed to crystallize with constant stirring. The crystals so obtained need not be pulverized.

A stock solution of trichloroacetic acid, made by dissolving one part of acid by weight with 1·5 parts of water, is stored in the ice box and diluted one to ten as needed. As Wendel [1931–32] pointed out, this reagent may have a high blank. Baker's CP product usually has a blank no higher than that of distilled water, although some samples have a small, but not troublesome, blank. Trichloroacetic acid can be purified by sublimation, but this is a wasteful procedure.

Method

The tissue grinder described by Potter & Elvehjem [1936] was found by Newman [1938] to be well suited for quantitative extraction of lactate from muscle. It consists of a pyrex test tube of 30 ml. capacity, graduated at 25 ml. and fitted with a revolving glass pestle that has a 15 cm. glass shaft on its upper end.[1] The shaft fits by means of a rubber stopper to the shaft of a 1/50 H.P. electric motor that has a built-in reducing gear of $8\frac{1}{2}$ ratio and a suitable rheostat.

A tube is set in a pan of solid CO_2 and 2 ml. of 4 % trichloroacetic acid are frozen in the bottom. The muscle is frozen in powdered CO_2 and a piece weighing

[1] These may be bought from Macalaster-Bicknell Co., Cambridge, Mass., U.S.A.

about 250 mg. is weighed, while frozen, with a rapid torsion balance. It is dropped into the tube, covered with 3 ml. of ice-cold 4 % trichloroacetic acid and the whole mass is frozen solid. The tube is dipped into cold water until melting just begins, the pestle is inserted, the motor started and the muscle is ground to a fine frozen pulp. This takes about 2 min. The pestle is withdrawn and washed off quantitatively into the tube with ice-cold 4 % trichloroacetic acid. The tube is filled to 25 ml. with the same reagent, a clean rubber stopper is inserted and the mixture is shaken. It is then centrifuged for 5 min. at about 2500 r.p.m.

A suitable aliquot of the supernatant fluid is taken for lactate estimation according to Edwards [1938], with certain precautions. The water leaving the condensers should range from 30° to 40°. Below 30° aldehyde is retained; above 40° aldehyde is lost. This has been pointed out by Friedemann & Kendall [1929] and by Friedemann & Graeser [1933]. They have also advised cooling the tube in which the titration takes place when the room temperature is above 26°. Otherwise blanks are high and recoveries of standard lactate low. It is for this reason that we use completely hydrated disodium phosphate which has a negative heat of solution [Lehnartz, 1928].

Edwards [1938] found that as many as eleven runs could be made without cleaning out the apparatus or adding fresh manganous sulphate and H_2SO_4 between runs. We have extended his findings on this point to muscle. Friedemann *et al.* [1927] stated that for relatively small amounts of lactate, such as are found in 200 mg. of muscle, satisfactory results can be obtained without the use of manganous sulphate, provided that the rate of oxidation is carefully adjusted.

In our battery of 10 units, each run contains a reagent blank, a standard of lithium lactate and 4 muscle extracts in duplicate. The recovery of the standard is always in the range 97–102 % of the theoretical amount. The blank is usually less than 0·04 ml. of 0·005*N* iodine, and rarely as much as 0·06 ml. Duplicates of the extracts always agree to within 0·02 ml. of 0·005*N* iodine. These blanks and recoveries compare favourably with Wendel's [1931–32], although our blanks are slightly higher than his. He got recoveries of only 95 % of the theoretical values when adding zinc lactate, which was thought to be impure. The sensitivity of the method in our hands is therefore about the same as in his, namely, down to about 0·01 mg. of lactic acid, corresponding to not more than 5 mg. of lactic acid per 100 g. of muscle, with duplicate estimations agreeing to within 2 mg. of lactic acid per 100 g.

By the present method, duplicate estimations on each of the 4 gastrocnemii of two frogs, along with a reagent blank and a standard solution, can be finished in from 70 to 90 min. after the animals are killed. With an accumulation of samples, one run is made while another is being titrated, so that 10 titrations can be finished every 30–40 min., and since cleaning out the apparatus, or adding fresh reagents, is unnecessary, there is no delay in beginning new runs.

Evidence justifying the various steps of the method

In methodological studies, it is necessary to have a supply of material so homogeneous that the concentration of the substance in question is constant from one sample to another. In the case of muscle, this is best achieved by powdering the muscle. We have used pills made from frozen frog muscle. In the course of preparation, all the equipment is cooled with solid CO_2. The muscles are frozen and powdered in a mortar and the powder is sifted first through a coarse and then a fine sifter. The pills are made in a pill press so constructed that they are hammered solid by a steel plug fitting into a hole bored through a block

of steel. They weigh between 200 and 300 mg. Duplicate estimations are made on each pill. The pills can be stored for days in airtight containers surrounded by solid CO_2 without change in content of water, glycogen or lactate. The lactate concentration is satisfactorily constant from pill to pill, the maximum difference in any given batch being 5 mg./100 g.

In taking an aliquot from the supernatant fluid, the assumption is made that all of the lactate originally present in the muscle is distributed in equal concentration by volume throughout both the supernatant fluid and the mass of denatured protein. Experiment 1 (Table 1) shows that in the present method this assumption is valid. The experiments were carried out on pills of the same batch, divided into two groups. The first group of analyses was made on aliquots taken from the supernatant fluid. The pills of the second group were ground up and the supernatant fluid was saved. Then each pill residue was extracted with three 5 ml. portions of the trichloroacetic acid. The original supernatant fluid and the washings were pooled, made up to known volume, and aliquots taken for analysis. Data of Newman [1938] are also included. The same result was obtained by these two methods of lactate extraction.

The three reagents most commonly used for deproteinizing muscle are trichloroacetic acid, mercury salts and sodium tungstate. Trichloroacetic acid in concentrations varying from 4 to 10 % has been used by Cori & Cori [1932–33], Davenport & Davenport [1928], Flock *et al.* [1939], Gordon & Quastel [1939],

Table 1. *Various factors influencing the estimation of muscle lactate*

Results for solutions of lithium lactate are expressed as mg. lactic acid per 100 ml., and are the average of duplicate or triplicate estimations.

Results for muscle and muscle pills are expressed as mg. lactic acid per 100 g. tissue, and are the average of duplicate or triplicate estimations on each of two or three samples.

Type of sample and treatment	Lactate content mg./100 ml. or g.	Lactate added mg./100 ml. or g.	Lactate recovered mg./100 ml. or g.	Lactate recovered % of amount added
Exp. 1				
Batch 3 of muscle pills: quantitative extraction	55	—	—	—
Batch 3 of muscle pills: aliquots of supernatant	55	—	—	—
Batch 4 of muscle pills: quantitative extraction	48	—	—	—
Batch 4 of muscle pills: aliquots of supernatant	49	—	—	—
Rat muscle 1: quantitative extraction	42	—	—	—
Rat muscle 1: aliquots of supernatant	38	—	—	—
Rat muscle 2: quantitative extraction	47	—	—	—
Rat muscle 2: aliquots of supernatant	43	—	—	—
Rat muscle 3: quantitative extraction	39	—	—	—
Rat muscle 3: aliquots of supernatant	40	—	—	—
Exp. 2				
4 % trichloroacetic acid:				
No added lactate	49	—	—	—
Lactate added	76	28	27	96·5
Lactate added	102	52	53	102
Quantitative extraction with acid sodium tungstate, followed by treatment with copper-lime:				
No added lactate	48	—	—	—
Lactate added	96	48	48	100
Quantitative extraction with acid mercuric sulphate followed by treatment with copper-lime:				
No added lactate	47	—	—	—
Lactate added	99	53	52	98

Table 1 (*cont.*)

Type of sample and treatment	Lactate content mg./100 ml. or g.	Lactate added mg./100 ml. or g.	Lactate recovered mg./100 ml. or g.	Lactate recovered % of amount added
Exp. 3				
Solutions of pure lithium lactate:				
In distilled water	—	1·91	1·91	100
In 4 % trichloroacetic acid	—	1·91	1·92	100·5
In 8 % trichloroacetic acid	—	1·91	1·89	99
In 16 % trichloroacetic acid	—	1·91	1·89	99
In distilled water	—	0·48	0·48	100
In 4 % trichloroacetic acid	—	0·48	0·49	102
In 8 % trichloroacetic acid	—	0·48	0·48	100
Pills of batch 4 ground in trichloroacetic acid:				
Ground in 10 times by wt. of 40 %, then diluted to 1 : 100 by wt.	54	—	—	—
Ground in 1 : 100 by wt. of 2 %	54	—	—	—
Ground in 1 : 200 by wt. of 2 %	58	—	—	—
Ground in 20 times its wt. of 4 %	54	—	—	—
Ground in 40 times its wt. of 4 %	53	—	—	—
Ground in 100 times its wt. of 4 %	57	—	—	—
Ground in 200 times its wt. of 4 %	55	—	—	—
Ground in 100 times its wt. of 8 %	57	—	—	—
Extracted quantitatively with 4 successive washings with 4 % trichloroacetic acid	55	—	—	—
Pills of batch 1 ground with 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	113	—	—	—
Added lactate	321	214	208	97
Pills of batch 2 ground with 100 times their wt. of 4 % trichloroacetic acid				
No added lactate	139	—	—	—
Added lactate	247	106	108	101·5
Pills of batch 5 ground in 100 times their wt. of 4 % trichloroacetic acid:				
No added lactate	49	—	—	—
Added lactate	111	61	62	101·5
Added lactate	73	24	24	100
Exp. 4				
4 % trichloroacetic acid extract of frog muscle, analysed with potassium permanganate by present method:				
No added lactate	383	—	—	—
Added lactate	591	211	208	98·5
Same extract, analysed with ceric sulphate according to Gordon & Quastel [1939]:				
No added lactate	383	—	—	—
Added lactate	698	320	315	98·5

Long & Grant [1930] and Long & Horsfall [1932] and others. Mercuric salts have been used by Lehnartz [1928], Kerly & Ronzoni [1937], Ronzoni & Wallen-Lawrence [1927], Ronzoni *et al.* [1928] and Lundsgaard [1933]. Sodium tungstate has been used much more with blood than with muscle.

Exp. 2 demonstrates that muscle extract is adequately deproteinized by any one of the foregoing methods. Pills of the same batch were divided into three groups. The first were ground and extracted with trichloroacetic acid, as described above. The second were treated with 1 ml. of 10 % sodium tungstate, 1 ml. of 2/3N H_2SO_4 and 8 ml. of water. After centrifuging, the supernatant

fluid was saved and the residues were extracted with three 5 ml. portions of the acid-tungstate mixture. The supernatant fluid and the washings were pooled, diluted to known volume and treated with copper sulphate and calcium hydroxide, after Van Slyke [1917]. The pills of the third group were extracted with four 5 ml. portions of 4 % mercuric sulphate in N H_2SO_4. The four extracts were pooled, treated with H_2S and centrifuged. The supernatant fluid was aerated until the H_2S was removed. Aliquots were made slightly alkaline with NaOH and treated with copper sulphate and calcium hydroxide. In each group, to certain pills lithium lactate in amounts comparable with the lactate already present was added. Exp. 2 shows that the estimated lactate was the same regardless of the type of protein precipitant, and also that recovery of added lactate was satisfactory. This has previously been shown for blood by Ronzoni & Wallen-Lawrence [1927], although they did not use trichloroacetic acid, by Edwards [1938] for blood and by Davenport & Davenport [1928] for muscle.

Table 2. *Comparison of results with and without preliminary aeration and treatment with copper-lime*

Results represent the averages of duplicate or triplicate estimations.

	No preliminary boiling and aeration		10 min. preliminary boiling and aeration	
Species	No Cu-lime mg./100 g.	Cu-lime mg./100 g.	No Cu-lime mg./100 g.	Cu-lime mg./100 g.
Frog	146	143	147	145
,,	41	39	40	39
,,	177	177	179	175
,,	13	11	11	13
Guinea-pig	99	—	—	103
Man	30	—	—	29
Rabbit	95	—	—	92
Rat	143	—	—	138

It is well known that trichloroacetic acid may be used in wide concentrations and amounts without affecting the recovery of added lactate so long as there is enough to precipitate all the protein [Cori & Cori, 1932–33; Avery *et al.* 1935]. Exp. 3 (Table 1), containing data obtained from solutions of pure lithium lactate and from muscle, further demonstrates that over a range of 4 to 16 % concentration of the reagent, lactate may be accurately estimated and recovery of added lactate is satisfactory. It should be emphasized that it is necessary to have trichloroacetic acid with a small blank before such consistently satisfactory results are obtained. We have fixed on 4 % trichloroacetic acid only because of its convenience, and have made up 250 mg. of muscle to a volume of 25 ml. only to avoid the error inherent in pipetting small volumes.

The evidence that with muscle, as with blood, preliminary boiling and aeration and treatment with copper sulphate and calcium hydroxide are unnecessary is obtained in Table 2. Extracts of several frog muscles were prepared with 4 % trichloroacetic acid along with suitable reagent blanks. One-half of each extract was treated with 10 % copper sulphate and 20 % calcium hydroxide. The proportions were 25 volumes of extract, with enough 40 % NaOH to make it neutral, 10 volumes of copper sulphate, 10 volumes of calcium hydroxide and water to make 50 volumes. The mixture was allowed to stand at least one-half hour, with shaking every 5 min. It was centrifuged and aliquots of the supernatant fluid taken for analysis. An analysis of each type of extract was made with and without the preliminary boiling and aeration that are customary. The

same results were obtained with and without treatment with copper and lime and with and without preliminary boiling and aeration. The table includes also data derived from the muscles of several other animal species. These data do not include estimation of the untreated extract with preliminary boiling, or estimation of the treated extract without preliminary boiling, since the results led to the same conclusion as those from frog muscle. The suggestion that treatment with copper and lime may be dispensed with under certain circumstances has been made by Friedemann *et al.* [1927], Friedemann [1928], Friedemann & Kendall [1929], Boyland [1928], Scott & Berg [1936] and Edwards [1938]. One of the advantages claimed for methods such as those used by Gordon & Quastel [1939], Lehmann [1938], Miller & Muntz [1938] and Rappaport & Reifer [1937], which differ in principle from that of Friedemann *et al.* [1927], is that removal of carbohydrates before analysis is unnecessary. We consider that with muscle it is not necessary even for the method of Friedemann *et al.* [1927].

We have tried repeatedly without success to detect in trichloroacetic acid extracts of frog muscles any appreciable amount of preformed volatile substances that will bind bisulphite.

Gordon & Quastel [1939] introduced the use of ceric sulphate instead of potassium permanganate as oxidant, claiming the advantage that treatment of muscle extracts with copper sulphate and calcium hydroxide is thereby rendered unnecessary. Exp. 4 compares their method with ours for the same muscle extract. The present method requires one-fourth of the time, employs simpler apparatus and gives the same results as that of Gordon & Quastel [1939].

Summary

1. The simplifications which Edwards [1938] introduced into the method and apparatus of Friedemann *et al.* [1927] for estimating lactate in normal human blood are applicable also to muscle. Treatment of muscle extracts with copper sulphate and calcium hydroxide, as well as preliminary boiling and aeration, may be dispensed with. From five to ten estimations can be made without cleaning out the apparatus or adding fresh reagents.

2. A rapid, accurate method for preparing protein-free extracts of muscle, which uses the tissue grinder of Potter & Elvehjem [1936], is described.

3. No difference in the estimated preformed lactate or in recovery of added lactate is found when either trichloroacetic acid, mercuric sulphate or acid sodium tungstate is used for deproteinizing.

4. The use of ceric sulphate as oxidant is no more precise or convenient than the use of potassium permanganate and requires more time and more complicated apparatus.

REFERENCES

Avery, Kerr & Ghantus (1935). *J. biol. Chem.* **110**, 637.
Boyland (1928). *Biochem. J.* **22**, 236.
Cori & Cori (1932–33). *J. biol. Chem.* **99**, 493.
Davenport & Davenport (1928). *J. biol. Chem.* **76**, 651.
Edwards (1938). *J. biol. Chem.* **125**, 571.
Flock, Ingle & Bollmann (1939). *J. biol. Chem.* **129**, 99.
Friedemann (1928). *J. biol. Chem.* **76**, 75.
—— Cotonio & Shaffer (1927). *J. biol. Chem.* **73**, 335.
—— & Graeser (1933). *J. biol. Chem.* **100**, 291.
—— & Kendall (1929). *J. biol. Chem.* **82**, 23.

Gordon & Quastel (1939). *Biochem. J.* **33**, 1332.
Kerly & Ronzoni (1937). *J. biol. Chem.* **103**, 161.
Lehmann (1938). *Skand. Arch. Physiol.* **80**, 237.
Lehnartz (1928). *Hoppe-Seyl. Z.* **179**, 1.
Long & Grant (1930). *J. biol. Chem.* **89**, 553.
—— & Horsfall (1932). *J. biol. Chem.* **95**, 715.
Lundsgaard (1933). *Biochem. Z.* **264**, 209.
Miller & Muntz (1938). *J. biol. Chem.* **126**, 413.
Newman (1938). *Amer. J. Physiol.* **122**, 359.
Potter & Elvehjem (1936). *J. biol. Chem.* **114**, 495.
Rappaport & Reifer (1937). *Microchim. Acta*, **2**, 62.
Ronzoni, Glaser & Barr (1928). *J. biol. Chem.* **80**, 309.
—— & Wallen-Lawrence (1927). *J. biol. Chem.* **74**, 363.
Scott & Berg (1936). *J. biol. Chem.* **115**, 163.
Van Slyke (1917). *J. biol. Chem.* **32**, 455.
Wendel (1931–32). *J. biol. Chem.* **94**, 717.

56. THE MICRO-DETERMINATION OF POTASSIUM

By C. R. HARINGTON

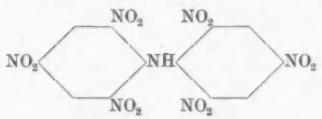
From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C.1

(*Received 26 March 1941*)

THE determination of potassium is more troublesome than that of any other biologically important cation; at the same time there are many conditions involving variations in the electrolyte content of the body fluids in which accurate estimations of potassium are desirable. It therefore appeared worth while to try to devise an analytical method for potassium which should be simpler than those at present in use; this paper describes a new method which offers certain advantages of simplicity whilst giving a degree of accuracy not significantly less than those claimed for the more time-consuming procedures.

The methods at present available for the determination of potassium fall into two groups: (1) determination as the chloroplatinate and (2) determination by one of the various modifications of the cobaltinitrite procedure [compare Peters & Van Slyke, 1932]. The chloroplatinate method is excellent for inorganic analysis when reasonably large quantities of material are available; it loses accuracy however when adapted to micro-analysis. The cobaltinitrite methods which have been most widely employed in biological work suffer from the theoretical disadvantage that there is uncertainty in the composition of the final precipitate; this means that although a series of analyses conducted by one worker under constant conditions may give mutually consistent results with a small margin of error, the basis of the method is not satisfactory.

In the search for a new procedure attention was directed to the possible use of dipicrylamine; this compound, di(2:4:6-trinitrophenyl)amine, forms a deep



orange-red potassium salt, of which the solubility in water is similar to that of potassium sodium cobaltinitrite; it has been proposed as a qualitative reagent for the detection of small amounts of potassium [Feigl, 1937] and is stated to be specific for potassium in presence of other metals of the 4th and 5th groups except rubidium and caesium and in absence of ammonium salts; no record has been found of its application to the quantitative determination of potassium.[1]

Preliminary experiments showed that potassium dipicrylamine was soluble in alcohol, to give, in low concentrations, solutions of a strong orange colour; this suggested the possibility of a determination of potassium based on measurement of the colour of an alcoholic solution of the precipitate of potassium dipicrylamine obtained from the solution to be analysed.

[1] Since this was written my attention has been called to two papers on the determination of potassium with the aid of dipicrylamine by Kolthoff & Bendix [1929] and Amdur [1940]; these papers do not however describe the application of the method to the analysis of blood.

Experimental

Colorimetric determination of potassium dipicrylamine

It was first necessary to ascertain the possibility of accurate measurement of the colour. For this purpose a series of standard solutions of potassium dipicrylamine in alcohol was prepared as follows. Commercial dipicrylamine was recrystallized from acetic acid; a solution of the sodium salt was made by dissolving the dipicrylamine (0·44 g.) in boiling $N/10$ sodium carbonate (20 ml.) and filtering the cooled solution; a slight excess of this solution was added to one of pure potassium nitrate containing 1 mg. K/ml.; after keeping overnight in the ice chest the potassium dipicrylamine was collected, washed with ice water and recrystallized from boiling $N/100$ potassium carbonate; the recrystallized material was dried *in vacuo* over sodium hydroxide. A stock solution of the salt in alcohol was made which contained 0·02 mg. K/ml. and from this a series of dilutions was prepared.

The colours of these solutions were measured in two ways: (*a*) with the Zeiss step-photometer; (*b*) with a simple photoelectric device [compare Harington *et al.* 1940]. Using the step-photometer, the best results were obtained with filter S 53, having maximum transmission at about 530 mμ; with the photoelectric device the most favourable conditions so far found have been with a combination of Wratten filter no. 61 and 2 mm. thickness of Chance's calorex glass to absorb the infra-red to which the cell is sensitive.

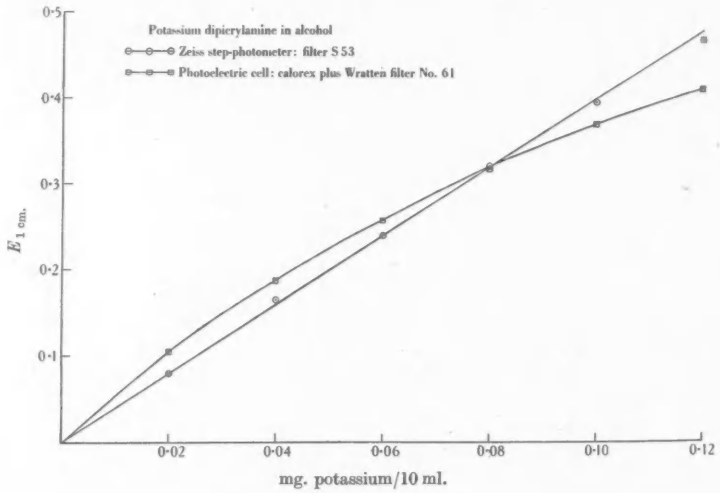


Fig. 1.

Results obtained by both these methods are shown in Fig. 1. It will be seen that over the range 0–0·1 mg. K/10 ml. the observations with the step-photometer are closely in accordance with Beer's law; at higher concentrations deviation from linearity became considerable. With the photoelectric device, on the other hand, a linear relationship between extinction coefficient and concentration of potassium is not obtained over any range; this is doubtless to be explained

by incomplete elimination of the infra-red and by the breadth of the transmission band of the Wratten filter employed.

It is clear that if the colour is to be measured photoelectrically it will be necessary to work from a calibration curve such as that given in Fig. 1; with the step-photometer on the other hand, provided that the solution is adjusted to give a value of $E_{1\,\mathrm{cm.}} < 0{\cdot}4$, the concentration of potassium is given by the formula

$$c = 2{\cdot}5E$$

where c is the concentration of potassium in mg./100 ml. in the final solution and E is the observed value of the extinction coefficient using filter S 53 and a 1 cm. cell.

The orange colour of the alcoholic solution of potassium dipicrylamine is not suitable for simple comparative colorimetry; such colorimetry can however be improved with the aid of suitable filters and fair results have been obtained with a Leitz Duboscq type of instrument using Wratten filter no. 61.

In all the analyses recorded below the colour measurements have been made with the step-photometer.

Analysis of solutions containing known amounts of potassium

The general method of analysis is as follows. The solution containing potassium at a concentration of 0·1–0·25 mg./ml. and at approximately neutral reaction is mixed with excess of the precipitating reagent (sodium or lithium dipicrylamine: see below); when crystallization of the potassium salt is complete it is collected, washed and dissolved in alcohol, the volume of the final solution being adjusted so that the value $E_{1\,\mathrm{cm.}}$ observed in the step-photometer falls in the range 0·20–0·40; the colour is then measured.

(*a*) *Precipitating reagent.* In the earlier experiments this consisted of a solution of sodium dipicrylamine prepared as described above; later, for reasons which will appear, this was replaced by a similar solution of lithium dipicrylamine.

(*b*) *Time of precipitation.* When the precipitant is mixed with a solution containing potassium at about 0·1 mg./ml. concentration, crystallization of the potassium salt is best initiated by scratching the walls of the tube with a fine glass rod; the crystallization is completed in the ice chest. An experiment was performed to determine the time necessary for complete precipitation; the results are shown in Table 1, from which it appears that 2 hr. suffice; this period was therefore adopted as a routine.

Table 1. *Effect of time of precipitation on recovery of potassium*

Sample	Time in ice chest	K present mg.	K found mg.
A	1 hr.	0·200	0·188
B	2 hr.	0·200	0·198
C	3 hr.	0·200	0·193
D	Overnight	0·200	0·198

(*c*) *Analysis of known solutions of potassium nitrate.* A stock solution of pure potassium nitrate was prepared, containing 2 mg. K/ml.; from this a number of dilutions were made by an independent observer. The results obtained are shown in Table 2.

Table 2. *Analyses of solutions of potassium nitrate*

Sample	K present mg./100 ml.	K found mg./100 ml.	Error %
A	20·0	19·4	−3·0
B	21·6	22·4	+3·6
C	26·8	27·6	+4·3
D	38·0	36·3	−4·5
E	42·8	40·8	−4·7

From this it appears that the error in the determination on simple potassium nitrate solutions is within ±5 %.

(*d*) *Effect of other blood cations.* In order to study this point a series of solutions was analysed which all contained 20 mg. K/100 ml., but which differed in the amounts of Na, Ca and Mg which were present; the results are given in Table 3.

Table 3. *Effects of other blood cations on recovery of potassium*

Solution	Present mg./100 ml.				K found mg./100 ml.
	Na	K	Ca	Mg	
A	350	20	10	3	19·3
B	700	20	20	6	23·1
C	700	20	—	—	23·6
D	—	20	20	—	19·3
E	—	20	—	6	19·3

The recovery from solution A, which contains the amounts of the various cations found in normal blood serum is satisfactory; solution B on the other hand, in which all cations save K are doubled in concentration, gives a high result; a similarly high result is obtained with solution C containing no Ca or Mg but double the normal amount of Na, whilst correct results are obtained in presence of double the normal amounts of Ca and Mg when Na is absent (solutions D and E). It is clear that the greatest danger of interference comes from Na, and extension of the above experiment showed that such danger exists at levels of Na only slightly above the normal.

The above analyses were carried out with the sodium dipicrylamine precipitant in which the sodium ion concentration is $N/10$; it was evident that the danger of interference from sodium could be eliminated if a more soluble salt of dipicrylamine could be found; the lithium salt is in fact much more soluble than the sodium salt and a precipitating reagent was therefore prepared by dissolving 0·44 g. of dipicrylamine in 20 ml. of $N/10$ lithium carbonate; with the aid of this reagent it is possible to determine potassium accurately in presence of proportions of sodium greatly in excess of those found in biological fluids (Table 4).

Table 4. *Lithium dipicrylamine as precipitant; determination of potassium in presence of increasing amounts of sodium*

Solution	Na mg./100 ml.	K mg./100 ml.	K found mg./100 ml.
A	—	20·0	19·7
B	350	20·0	20·0
C	450	20·0	20·3
D	550	20·0	19·7
E	650	20·0	20·0

In view of these results the lithium dipicrylamine solution was adopted as precipitant in all future experiments.

Determination of potassium in blood serum

The analysis cannot be carried out directly on blood serum since the precipitation of potassium dipicrylamine is apparently inhibited by the organic constituents; the method adopted in the present work has been to incinerate a trichloroacetic acid filtrate of serum with sulphuric and nitric acids and to analyse the solution of the sulphate ash. This procedure has the advantages over dry incineration of greater rapidity and freedom from danger of loss of alkali chloride.

Reagents.

1. *Trichloroacetic acid*, 20 % aqueous solution.
2. *Sulphuric acid*, Analar, 4*N* solution.
3. *Nitric acid*, Analar, concentrated.
4. *Lithium dipicrylamine.* Commercial dipicrylamine is recrystallized from glacial acetic acid, 0·44 g. is dissolved by boiling in 20 ml. of *N*/10 lithium carbonate; the cooled solution is filtered.
5. *Potassium dipicrylamine*, saturated aqueous solution. The recrystallized salt is dissolved in warm *N*/1000 potassium carbonate in the proportion of 1 mg./ml. and the solution is kept in the ice chest until crystallization is complete; the mother liquor is used as washing fluid (see below).

Procedure.

In drawing the blood precautions must be taken to avoid haemolysis. 1 ml. serum is mixed with 3 ml. distilled water followed by 1 ml. 20 % trichloroacetic acid and the mixture is filtered. Of the filtrate 2·5 ml. (equivalent to 0·5 ml. serum) are placed in a 6 × 1 in. pyrex test tube together with 0·6 ml. 4*N* H_2SO_4 and a glass bead; the water is boiled off and the charred residue treated, after partial cooling, with one drop of concentrated HNO_3; heating is then resumed until the residue is colourless; the whole process is complete in a few minutes. The residue is then transferred quantitatively with small amounts of water to a platinum dish and evaporated as far as possible on the steam bath; excess of sulphuric acid is removed by cautious heating over the free flame, the contents of the dish being finally briefly but strongly ignited.

The ash is transferred with small quantities of water to a 3 × $\frac{3}{8}$ in. test tube and the solution evaporated to 0·5–1·0 ml.; 1 ml. of the lithium dipicrylamine solution is added and crystallization of the potassium salt is initiated by scratching the tube with a fine glass rod. After 2 hr. in the ice chest the precipitate is collected on a small sintered glass funnel (1·0 cm. disk; porosity G 3 or G 4), the precipitation tube being carefully drained, and the precipitation tube and funnel are washed with two 1 ml. portions of the ice-cold saturated aqueous potassium dipicrylamine and finally with 1 ml. of ice-cold distilled water.

The receiver is then changed and the precipitation tube and funnel are washed through with warm alcohol, which dissolves the potassium salt; the alcoholic filtrate is made up to 15 ml. and the colour measured in the step-photometer. With normal samples of serum this will give a value of $E_{1\,\mathrm{cm.}}$ between 0·24 and 0·32.

The results of a series of determinations on the sera of normal individuals are given in Table 5; the range observed, 17·5–24 mg./100 ml., is in accordance with the accepted values for normal serum potassium.

Table 5. *Potassium content of normal blood serum*

Sample no.	K found mg./100 ml.	Sample no.	K found mg./100 ml.
1	20·7	8	19·2
2	18·1	9	17·5
3	18·5	10	18·5
4	18·1	11	20·0
5	18·8	12	24·0
6	19·0	13	22·5
7	18·8	14	21·0

Recovery of potassium added to serum

In Table 6 are recorded the results of experiments in which analyses were made of a sample of serum before and after addition of measured amounts of potassium.

Table 6. *Recovery of potassium added to serum*

Sample no.	K added mg./100 ml.	K found mg./100 ml.	K recovered mg./100 ml.	Error %
1	0	20·7	—	—
2	10	31·3	10·6	+6·0
3	20	42·0	21·3	+6·5
4	40	59·5	38·8	−3·0

It will be seen that recoveries are satisfactory.

Summary

A method for the determination of potassium is described which is based on precipitation as potassium dipicrylamine; the precipitate is collected, washed and dissolved in alcohol, and the potassium content determined by measurement of the colour of the resulting solution.

The application of the method to determination of blood serum potassium is described.

REFERENCES

Amdur (1940). *Industr. engng. Chem.* Anal. Ed. **12**, 731.

Feigl (1937). Spot Tests, p. 144. New York: Nordemann Publishing Company.

Harington, Pochin & Squire (1940). *Clin. Sci.* **4**, 311.

Kolthoff & Bendix (1929). *Industr. engng. Chem.* Anal. Ed. **11**, 94.

Peters & Van Slyke (1932). Quantitative Clinical Chemistry, **2**, 741–55. London: Baillière, Tindall & Cox.

57. ESTIMATION OF VITAMIN A AND CAROTENE IN HUMAN BLOOD

By SIMON YUDKIN

From the Department of Chemical Pathology, University College Hospital Medical School, London, W.C.1

(*Received 31 March 1941*)

During the past few years numerous investigations have been carried out on the vitamin A and carotene contents of human blood in health and various diseases. Unfortunately, the method of estimation varies from author to author and in many cases the results are given in other than international units [Dost, 1937; Kimble, 1939; May *et al.* 1940], so that comparison is impossible. The following investigation was undertaken with a view to discovering the relative efficacies of the various methods of estimation. A preliminary series of normal blood values is also given.

Discussion of methods

The extraction is usually done on serum or plasma. Many authors saponify the serum with alcoholic KOH, some in the cold to eliminate the effect of haemolysis [Willstaedt & With, 1938], others on a boiling water bath for longer [Lindquist, 1938] or shorter [Pett & Le Page, 1940] periods. Clausen & McCoord [1938] and Kimble [1939] extract directly from serum precipitated with alcohol. The extraction is done either with ether or light petroleum and the resulting extract evaporated under nitrogen or carbon dioxide or in air.

The estimation of the vitamin A is usually carried out by means of the blue reaction of Carr and Price, but the absorption at 328 mμ is sometimes estimated directly [Chevallier & Choron, 1935]. The depths of the yellow colour of the carotene and the blue colour of the vitamin A reaction have been estimated in the following ways.

1. *Comparison in colorimeter with standard solutions* (potassium dichromate for carotene and either copper sulphate or a mixture of copper sulphate and cobalt nitrate [Schneider & Widmann, 1935] for the blue colour). The main criticism of this method is the fact that the colours of standard and unknown are not spectrographically the same. (Indeed 10 % copper sulphate solution may be used as part of the filter system in photoelectric colorimetry of the blue colour.) Besides this the rapid fading of the blue colour makes matching difficult.

2. *Lovibond tintometry.* The same criticisms apply (see Notevarp & Weedon [1936] for more detailed discussion of these points).

3. *The Zeiss step-photometer* is more accurate and fairly satisfactory but again colour matching is difficult because of fading.

4. *Spectrophotometry* at 328 mμ is accurate but needs complicated apparatus and there are several interfering factors at this wave-length.

5. *Photoelectric colorimetry.* This method is at present the most satisfactory. It eliminates the personal factor of colour matching and makes it easy to estimate the density of the blue colour at its real maximum. It should, however, be remembered that the solutions do not necessarily follow Beer's Law [Koehn & Sherman, 1940], so that a calibration curve or table must be constructed. The

best method of estimation would be photoelectric colorimetry with monochromatic light (a 'monochromator') but there is, as far as I know, no such instrument available.

For the present investigation a simple photoelectric colorimeter was therefore used, with combinations of filters with maximum transmission at 440 mμ for the carotene and 620 mμ for the blue colour of the Carr-Price reaction (Fig. 1).

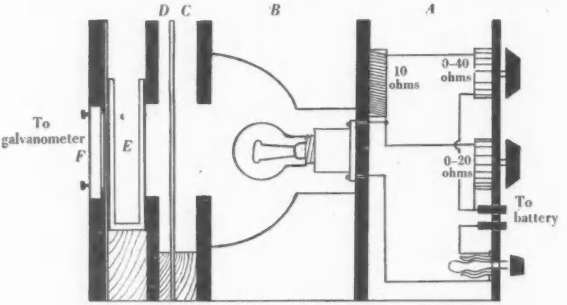


Fig. 1. Simple photoelectric colorimeter.

A. Simple circuit to give coarse and fine adjustment of illumination.

B. Source of illumination. 6 V. 3 W. Mazda lamp with small U-filament, run off four 2 V. cells to give increased proportion of blue light for the 440 mμ filter. Ordinary hemispherical torch reflector behind.

C. Space for filters. Filter 620 mμ maximum; Wratten 26 red gelatin filter combined with 1 mm. thickness of 'calorex' glass and 1 mm. thickness of 'signal green' glass, manufactured by Messrs Chance. Filter 440 mμ maximum; Wratten gelatin filters 35 and 2A combined with 2 mm. thickness of 'signal green' glass.

D. Space for card to cut off all light falling on photocell when not in use. Thin glass plate between this and filters.

E. Glass cell holding test solutions. Volume about 4 ml. depth 10 mm. optically ground. Marked on edge at 0·3 ml. and 3·3 ml.

F. Photocell. Selenium barrier cell manufactured by Mr Wender. Size 1 cm. × 2 cm. Thin glass plate in front.

Galvanometer; mirror type; sensitivity 12 cm. deflexion at 1 m. per microamp. (Messrs Pye Ltd.).

A smaller glass cell and smaller photoelectric cell may be used. The volumes used in these experiments are the most suitable for the sizes given.

Calibration

Carotene. A solution of carotene in arachis oil[1] containing 200 international units (120 μg.) per g. was used. A weighed quantity was dissolved in light petroleum and aliquots made up to the 3·3 ml. mark in the standard cell. The deflexions on the galvanometer from the '~~centre position~~' were noted and the calibration table constructed (Table 1). blank reading of 100

Vitamin A. A solution was made of about 10 mg., accurately weighed, of pure vitamin A 2-naphthoate[1] in 25 ml. pure arachis oil containing a trace of quinol. 5 ml. of this solution were made up to 50 ml. with pure dried chloroform and aliquots of this were used for the Carr-Price reaction. (The actual volumes were aliquots made up to 0·3 ml. and 3 ml. of reagent added, but these depend on the size of the cell in the colorimeter) (Table 2).

Correction for blue colour produced by carotene. A solution of a weighed quantity of carotene was made in chloroform. Aliquots were made up to 0·3 ml.

[1] Kindly supplied by British Drug Houses Ltd., London.

and 3 ml. of antimony trichloride reagent were added in the colorimeter with the 620 mμ filter in place. The readings were noted and the calibration table constructed (Table 3).

Table 1. *Calibration table for pure β-carotene*

Filter 440 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total μg. in solution	Log I_0/I per μg.
0·05	92·5	0·0339	0·70	0·0487
0·1	85·5	0·0680	1·40	0·0485
0·2	74	0·1308	2·79	0·0468
0·3	64·5	0·1904	4·17	0·0454
0·4	57·5	0·2403	5·58	0·0431
0·5	51	0·2924	6·98	0·0419
0·6	45	0·3468	8·37	0·0414
0·7	41	0·3872	9·77	0·0397
0·8	38	0·4202	11·16	0·0377
0·9	35·5	0·4498	12·56	0·0367
1·0	33	0·4815	13·95	0·0345

Table 2. *Calibration table for vitamin A 2-naphthoate*

Filter 620 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total international units in solution	Log I_0/I per international unit
0·025	87	0·0648	2·20	0·0295
0·05	75·5	0·1264	4·40	0·0292
0·075	65	0·1914	6·61	0·0290
0·10	56	0·2561	8·81	0·0290
0·125	49	0·3141	11·01	0·0285
0·15	44	0·3608	13·21	0·0274
0·2	35·5	0·4553	17·62	0·0260
0·25	29·5	0·5360	22·02	0·0244
0·3	24·5	0·6151	26·43	0·0234

Vitamin A 2-naphthoate contains 2,225,000 international units vitamin A per g. [Mead *et al.* 1939].

Table 3. *Correction curve for blue colour produced by β-carotene*

Filter 620 mμ

Vol. of aliquot in ml.	Galvanometer reading mm.	Log I_0/I	Total μg. in solution	Log I_0/I per μg.
0·025	98	0·0079	0·92	0·00873
0·05	96·25	0·0168	1·84	0·00920
0·075	94·5	0·0246	2·76	0·00894
0·10	93·5	0·0339	3·68	0·00920
0·125	91·0	0·0410	4·60	0·00893
0·15	89	0·0504	5·52	0·00913
0·20	86	0·0655	7·36	0·00892
0·25	82·5	0·0835	9·20	0·00908
0·30	79·5	0·0996	11·04	0·00903

Comparison of methods for extraction from blood

Multiple samples of serum or plasma were extracted by each of the following four methods:

1. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with ether ('alkali digestion method') [De Haas & Meulemans, 1938; Lanzing, 1938; Van Eekelen & Emmerie, 1934]. (For details see Lindquist [1938].)

2. Preliminary saponification with alcoholic KOH on a boiling water bath followed by extraction with light petroleum [Pett & Le Page, 1940].

3. Extraction three times with ether after precipitation with alcohol; no saponification.

4. Extraction by light petroleum, after precipitation with alcohol; no saponification [Kimble, 1939; May *et al.* 1940; Clausen & McCoord, 1938]. (For details see later.)

The results are given in Table 4.

Table 4

Extraction of serum or plasma by different methods; results in μg. per 100 ml. for carotene and international units per 100 ml. for vitamin A.

		Saponification followed by				Simple extraction with			
		Extraction with ether		Extraction with light petroleum		Ether after precipitation with alcohol		Light petroleum after precipitation with alcohol	
Subject	Date	Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A	Carotene	Vitamin A
V. K. C.	9. i. 41	149	99	108	78	149	102	153	102
A. C.	11. i. 41	135	115	71	50	135	115	135	139
S. L.	22. i. 41	102	157	73	144	102	157	102	157
I. L.	3. ii. 41	118	180	76	132	118	152	116	169
J. D.	11. ii. 41	154	129	140	121	140	154	154	155
M. S.	14. ii. 41	151	150	106	152	113	161	140	161
J. S.	21. ii. 41	116	150	65	130	140	145	121	134
A. B.	24. ii. 41	124	140	40	84	94	140	134	138
B. C.	5. iii. 41	104	125	67	98	134	122	134	130
B. W.	5. iii. 41	50	96	27	90	50	118	66	128
C. D.	21. iii. 41	50	96	34	50	50	115	59	101
Pooled serum	10. ii. 41	170	381	76	234	170	370	160	375

It can be seen from Table 4 that saponification followed by extraction with light petroleum gives consistently lower results than any other method. (Incidentally I could not confirm that saponification for a longer time than 5 min. (see Pett & Le Page [1940]) had any effect on the final result.) Simple extraction either with ether or petroleum after precipitation gives results as good as or better than those obtainable by Lindquist's more complicated method. As the simple light petroleum extraction is the easiest of all it is used for the estimation in normal sera.

Procedure for estimation in blood

3 ml. of serum or plasma are put into a narrow-necked tube. 3 ml. of 95 % alcohol are added slowly with shaking, followed by 6 ml. of pure light petroleum (B.P. 40–60°) and the tube tightly corked. It is then shaken by hand for 10 min. The tube is centrifuged for 1 min. at low speed to separate the layers. The light petroleum layer is removed into another tube and a 5 ml. aliquot taken. This is evaporated in a gentle stream of carbon dioxide or nitrogen in a water bath at 30–40° and then at 70° for a few seconds. The residue is taken up in light petroleum and made up to 3·3 ml. in the standard cell. The deflexion of the galvanometer from the ~~centre position~~ blank readings, of ice, with the 440 mμ filter in place is noted and the result expressed in μg. of carotene per 100 ml. of serum. The solution is evaporated to dryness under carbon dioxide or nitrogen and the residue dissolved in chloroform and made up to the 0·3 ml. mark. The 620 mμ filter is put in and the light altered to the 'centre position'; the cell is put in and 3 ml. of antimony trichloride reagent added all at once. The reading at the point of temporary stability is noted and after correction for the amount of blue colour produced

by the carotene, the vitamin A is expressed in international units per 100 ml. of serum.

Notes. 1. The 'centre position' is found as follows. The cell containing pure petroleum up to 3·3 ml. is put in with the 440 mμ filter. The light is altered to give a reading of 100. The reading when the cell is removed is the 'centre position' for the 440 mμ filter. Similarly 0·3 ml. of chloroform and 3 ml. of antimony trichloride reagent are used with the 620 mμ filter.

2. The alcohol must be added slowly and the mixture shaken while adding. Also vigorous shaking by hand must be continued for at least 10 min. Estimations of the extract after different periods of shaking show that with the alcohol added slowly almost complete extraction occurs after 5 min., but when it is added all at once a tough precipitate forms which does not yield the maximum readings until shaking for 20 min. has been carried out. Mechanical shaking on a machine also gives low readings. Evaporation of the extract with air destroys some of the vitamin A but leaves the carotene unharmed.

3. The yellow pigment measured in serum extracts is not all carotene. The proportion of the different pigments, however, is not easy to discover and it varies from serum to serum. The correction for carotene is applied on the assumption that all the yellow colour is due to carotene. Even if only half is carotene the difference would usually be only about 10 or 15 units of vitamin A.

To check the efficiency of the method several further tests were made.

1. The residue after removal of the light petroleum was re-extracted with a further 6 ml. of light petroleum. The results were never higher than 5 μg. of carotene and 6 units of vitamin A per 100 ml. serum.

2. Duplicate samples never gave a difference of more than 2 in the galvanometer readings. This corresponds to an error of about ±3 %.

3. Extractions of different volumes of the same serum gave the same final results.

4. Slight degrees of haemolysis had no effect on the results.

5. Storage of the serum for times up to 48 hr. in a refrigerator at 2° had no effect and the results were the same as the estimations on fresh serum.

Table 5. *Normal values*

Subject	Date	Carotene in μg. per 100 ml. serum or plasma	Vitamin A in international units per 100 ml. serum or plasma
1	12. vii. 40	151	132
2	22. vii. 40	150	112
3	27. vii. 40	220	157
4	27. vii. 40	182	136
5	27. vii. 40	241	122
6	29. vii. 40	158	135
7	29. vii. 40	161	100
8	15. viii. 40	117	112
9	12. viii. 40	117	136
10	28. viii. 40	130	107
11	6. ix. 40	117	84
12	20. ii. 41	90	110
13	20. ii. 41	104	102
14	19. iii. 41	118	151
15	19. iii. 41	58	94
16	19. iii. 41	98	103
17	19. iii. 41	67	120
18	20. iii. 41	63	100
19	20. iii. 41	82	72
20	20. iii. 41	90	121
21	25. iii. 41	94	97
22	25. iii. 41	104	86
23	25. iii. 41	50	103

The recovery from serum of added vitamin A and carotene is not admissible as evidence of accuracy as there is no relation between the form of vitamin A and carotene already present in the serum and that in which it is added.

There follows a series of values on normal healthy volunteers between 18 and 30 years of age (Table 5). They were all tested on the dark adaptation tester described by the author [Yudkin, 1941] and only those normal by this test were used.

The number of cases is too small to draw any definite conclusions though it is of interest to note that those estimated in February and March are in general lower than those done in the autumn, a possible seasonal effect. The range in general is similar to that of Lindquist [1938] and Kimble [1939].

Summary

The methods of estimating vitamin A and carotene in human plasma are discussed. The reasons for using a photoelectric colorimeter for the measurement of the colours are given.

A parallel series of results obtained by the use of four different methods of extraction shows that the simple extraction from serum or plasma precipitated with alcohol is the most satisfactory.

A preliminary series of normal readings is given.

I would like to thank the many students and others who offered themselves for the experiments, and Prof. C.R. Harington for his kind interest and for the laboratory and other facilities for this work.

REFERENCES

Chevallier & Choron (1935). *C.R. Soc. Biol., Paris*, **118**, 889.
Clausen & McCoord (1938). *J. Pediat.* **13**, 635.
De Haas & Meulemans (1938). *Geneesk. Tijdschr. Ned.-Ind.* **78**, 847.
Dost (1937). *Klin. Wschr.* **16**, 273.
Kimble (1939). *J. Lab. clin. Med.* **24**, 1055.
Koehn & Sherman (1940). *J. biol. Chem.* **132**, 527.
Lanzing (1938). *Geneesk. Tijdschr. Ned.-Ind.* **78**, 3135.
Lindquist (1938). *Acta. med. scand.* **97**, Supplement.
May, Blackfan, McCreary & Allen (1940). *Amer. J. Dis. Child.* **59**, 1167.
Mead, Underhill & Coward (1939). *Biochem. J.* **33**, 589.
Notevarp & Weedon (1936). *Biochem. J.* **30**, 1705.
Pett & Le Page (1940). *J. biol. Chem.* **132**, 585.
Schneider & Widmann (1935). *Klin. Wschr.* **14**, 670.
Van Eekelen & Emmerie (1934). *Acta. brev. neerl. Physiol.* **4**, 171.
Willstaedt & With (1938). *Nord. med. tidskr.* **15**, 648.
Yudkin (1941). *Brit. J. Ophthal.* **24**, 231.

58. STUDIES IN DETOXICATION

10. THE CONJUGATION AND OXIDATION OF *p*-HYDROXYBENZENESULPHONAMIDE IN THE RABBIT. THE CHARACTERIZATION OF *p*-SULPHONAMIDOPHENYLGLUCURONIDE

By H. G. SAMMONS,[1] JEAN SHELSWELL[1] and R. TECWYN WILLIAMS

From the Physiology Department, Medical School, Hospitals Centre, Birmingham, 15

(*Received 5 March 1941*)

Investigations in progress in this Laboratory on the fate of sulphonamide drugs in the animal body indicate that these are oxidized to a small extent with the production of phenolic substances which may be excreted in conjugation with sulphuric and glucuronic acids [cf. Shelswell & Williams, 1940; Thorpe & Williams, 1940; 1941; Thorpe *et al.* 1941]. In addition to this Scudi [1940] has isolated from the urine of dogs treated with sulphapyridine, the conjugated glucuronide of a hydroxysulphapyridine. It therefore became necessary to study the fate of an aryl sulphonamide containing a phenolic hydroxyl group, since such a study might be of some help in identifying the phenolic substances occurring in urine after the ingestion of sulphanilamide and related drugs. *p*-Hydroxybenzenesulphonamide (hereafter called phenol-*p*-sulphonamide) was selected as it was easily prepared and was also the simplest compound of the type required for study. No previous study has been made of its fate in the animal body, but Buttle *et al.* [1936], among others, have shown that it is therapeutically inactive.

The conjugation and oxidation of phenolsulphonamide

The present work shows that this sulphonamide when fed to rabbits is mainly excreted in the urine conjugated with glucuronic and sulphuric acids; in addition to this a very small portion is oxidized to a catechol derivative, possibly catechol-sulphonamide. No free phenolsulphonamide was detected in the urine, since ether extraction of the urine as such (faintly alkaline in reaction) and after acidification gave no trace of the sulphonamide. After hydrolysis with HCl, however, phenolsulphonamide together with the catechol compound could easily be extracted with ether.

The amount of phenolsulphonamide excreted conjugated with sulphuric acid depends upon the dose fed, the percentage conjugation falling with increasing dose (see Table 1). At the smallest dose level used (100 mg./kg.) the sulphate conjugation is more than 50 % of the dose, falling to less than 30 % at the highest level (750 mg./kg.). This behaviour recalls that of phenol, whose sulphate conjugation at various dose levels was studied by one of us [Williams, 1938]. Baumann & Herter [1877–8] have shown that the corresponding acid, phenol-*p*-sulphonic acid, produced no increase in ethereal sulphate excretion in the dog.

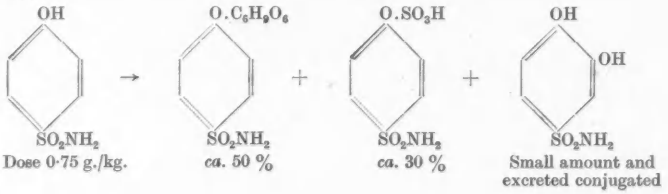
[1] Assisted by grants from the Caroline Harrold Research Fund, University of Birmingham.

Table 2 shows that this is also the case for the rabbit. In addition to phenol-*p*-sulphonic acid (Na salt, B.D.H.) other phenolic sulphonic acids were also fed, namely, 1-hydroxy-2-amino- (B.D.H.), 1-amino-2-hydroxy- [Friedländer, 1911] and 1-amino-3-hydroxy-benzene-4-sulphonic [Friedländer, 1896] acids. None of these acids caused the formation of ethereal sulphate in the rabbit (see Table 2), although they possess a phenolic OH group. A similar result was obtained by Williams [1938] with salicylic acid. Neither was the formation of conjugated glucuronic acid detected (by the naphthoresorcinol reaction) after feeding these acids. Other forms of conjugation were not tested for, as they were very unlikely to occur with the above compounds. It seems very probable that these sulphonic acids are excreted unchanged by the rabbit. Table 2 also includes toluene-*p*-sulphonamide, which causes no increase in ethereal sulphate formation in the rabbit. This compound is of interest since Flaschenträger *et al.* [1934] have shown that about 31 % of it is oxidized to *p*-sulphonamidobenzoic acid in the dog.

Judging from the maximum amount of barium sulphonamidophenylglucuronate that could be isolated from rabbit urine after administration of the sulphonamide, some 50 % is excreted conjugated with glucuronic acid, the dose being 0·75 g./kg. Therefore when the dose is 0·75 g./kg., over 80 % of the phenolsulphonamide can be accounted for by the conjugated glucuronide and ethereal sulphate excreted in the urine.

It was found that crude specimens of the above barium salt gave a green colour with $FeCl_3$ in aqueous solution. This colour was not due to free phenolsulphonamide since the latter gives a feeble purple colour with $FeCl_3$ which is discharged by acid or alkali. The alcoholic filtrates from the preparation of the barium salt (see experimental section) were evaporated to a syrup. The syrup was water-soluble and reduced ammoniacal silver nitrate but not Fehling's solution. Its aqueous solution gave an intense green colour with $FeCl_3$ which turned to blue-green, purple and then red on making the solution progressively more alkaline with Na_2CO_3. Acid discharged the colour. These reactions are similar to those of catechol. Further investigation showed that the syrup was mainly *p*-sulphonamidophenylglucuronide containing a small amount of a catechol derivative which was responsible for the $FeCl_3$ test. This catechol compound was excreted in conjugation since the urine itself gave no $FeCl_3$ reaction nor could a $FeCl_3$ reaction be obtained on ether extracts of the urine at acid or faintly alkaline reactions. The substance could be extracted together with phenolsulphonamide from acid-hydrolysed urines. Several attempts were made to isolate and identify this compound, but owing to the smallness of the amount present, the attempts have so far been fruitless. Such attempts, however, are being continued in conjunction with other work of a similar nature. The very characteristic $FeCl_3$ test and the reduction of ammoniacal $AgNO_3$ leave no doubt in our minds that a catechol derivative is present.

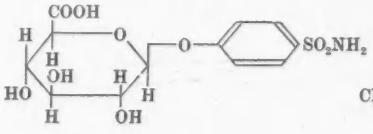
The fate of phenol-*p*-sulphonamide in the rabbit can therefore be summarized as follows:



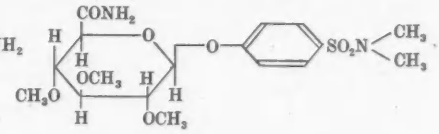
The formation of the above catechol derivative is an example of biological oxidation of the benzene ring in the *o*-position to an existing —OH. Baumann & Preusse [1879] have shown that this *ortho* oxidation takes place during the oxidation of phenol in the dog, since they found small amounts of catechol and quinol in the urine after larger doses of phenol. Biological oxidation can also take place *ortho* to an $—NH_2$, since *o*-aminophenols are produced in animals from a number of aromatic amino-compounds, e.g. formanilide [Kleine, 1896–7], acetanilide and *o*-acetotoluidide [Jaffe & Hilbert, 1888] and dimethylaniline [Horn, 1936]. There is also the case of β-naphthylamine which is oxidized in rabbits to β-amino-α-naphthol [Wiley, 1938]. A similar oxidation is possible with sulphanilamide [cf. Thorpe *et al.* 1941], since the formation of a catechol derivative from phenolsulphonamide suggests that biological oxidation can take place not only in a position *ortho* to the —OH group but also *meta* to the sulphonamide group.

The characterization of p-*sulphonamidophenylglucuronide*

Removal of barium from barium sulphonamidophenylglucuronate gave the free conjugated glucuronic acid as a brown uncrystallizable syrup. Many phenolic glucuronides behave in this manner and they have often been characterized as barium salts. These salts, however, are usually amorphous and difficult to purify. We therefore subjected the above barium salt to methylation with methyl sulphate and alkali followed by methyl iodide and silver oxide. In this manner the *methyl ester* of *trimethyl* p-*sulphondimethylamidophenylglucuronide* was obtained as a syrup (the sulphonamide group being methylated as well as the sugar residue). Treatment of this ester with methyl alcoholic ammonia gave a crystalline *amide* (II) whereby the glucuronide (I) could be characterized:



(I)



(II)

Pryde & Williams [1933] have shown that, in the case of biosynthetic bornylglucuronide, the sugar ring is of the pyranoid type and, since the glycosidic link in biosynthetic glucuronides is of the β-type (for literature see Pryde & Williams [1934]), the glucuronide excreted by rabbits after administration of phenolsulphonamide appears to be *p*-sulphonamidophenyl-β-*d*-glucopyranuronoside (I).

Experimental

Phenol-*p*-sulphonamide was easily prepared by diazotization of sulphanilamide followed by heating of the acid diazo solution on a boiling water bath. The sulphonamide was extracted from the solution with ether. The yield was 38.g. from 50 g. sulphanilamide. The M.P. after recrystallization from water was 176° (lit. M.P. 176°).

The ethereal sulphate conjugation

The percentage of phenolsulphonamide excreted as ethereal sulphate was determined exactly as described in previous papers on the sulphate conjugation

of substituted phenols and sulphanilamide [Williams, 1938; Shelswell & Williams, 1940]. The results are given in Table 1.

Table 1.* *The conjugation of phenolsulphonamide with sulphate in the rabbit*

Rabbit no.	Dose mg./kg.	Amount of phenolsulphonamide conjugated with sulphate		
		%		mg./kg.
77	100	61·1		53
11		59·1	av. 53	
9		47·5		
76		44·9		
9	250	51·5		110
63		42·5	av. 44	
62		38·1		
9	500	38·3		165
76		34·9	av. 33	
70		26·8		
9	750	30·2		203
77		29·7	av. 27	
63		25·5		
76		23·9		

* The figures in this table will include any sulphate conjugated with the catechol derivative.

Table 2

Compound	Dose g./kg.	Sulphate conjugation
1-Hydroxybenzene-4-sulphonic acid	0·5	0
1-Hydroxy-2-aminobenzene-4-sulphonic acid	0·5	0
1-Amino-2-hydroxybenzene-4-sulphonic acid	0·5	0
1-Amino-3-hydroxybenzene-4-sulphonic acid	0·5	0
Toluene-*p*-sulphonamide	0·1	0

The glucuronic acid conjugation

Barium p-*sulphonamidophenylglucuronate.* Rabbits (2–3 kg.) were each given by stomach tube 2 g. of phenolsulphonamide dissolved in 10–20 ml. warm water. The urine was collected during 2 days. It was made faintly acid with glacial acetic acid and then treated with saturated normal lead acetate solution until precipitation was complete. The solution was filtered and, after neutralization by conc. ammonia, the clear yellow filtrate was completely precipitated with saturated basic lead acetate. The basic lead precipitate was collected on a Büchner funnel, thoroughly washed with water, and, before it had time to dry, was made into a thin cream with water. This suspension was gassed with H_2S and the PbS filtered off under suction. The filtrate was concentrated *in vacuo* to a dark syrup which failed to crystallize. The syrup was now dissolved in water and treated with an excess of $BaCO_3$ and the resulting mixture filtered and washed with water. The filtrate and washings (the volume was kept as small as possible) were now poured into a large volume of alcohol, thereby precipitating the required *barium salt.* Next day the salt was filtered, washed with alcohol or acetone and dried in a desiccator. The alcoholic filtrates from this salt were preserved for further investigation (see below). Purification of the salt was accomplished by repeated precipitation from aqueous solution by alcohol, followed by washing with acetone and drying off the acetone *in vacuo.* The barium salt so obtained was a faintly coloured electrostatic powder, soluble in water, giving a pale reddish-brown solution and an intense naphthoresorcinol

reaction. It showed $[\alpha]_D - 50°$ ($c = 1$, in water). (Found: Ba, 16·6; N, 3·1 %. $(C_{12}H_{14}O_9NS)_2Ba$ requires Ba, 16·5; N, 3·3 %.) The yield of the crude salt was 1–1·25 g./g. of phenolsulphonamide fed. Aqueous solutions of the crude salt gave an intense green colour with $FeCl_3$, but the pure compound gave no colour. Removal of the Ba and subsequent suitable treatment gave the free glucuronide as a dark hard resin.

Hydrolysis of the barium salt. The salt (1 g.) was dissolved in 10 ml. water and the barium quantitatively removed with $2N$ H_2SO_4. To the filtrate and washings (40 ml.) from the $BaSO_4$, 10 ml. conc. HCl were added, thus making the solution about 2·5 N with respect to HCl. The solution was now gently boiled under reflux for 3 hr. and, after cooling and filtering to remove some black insoluble material, the strongly reducing solution was exhaustively extracted with ether. Removal of the ether left a sticky crystalline residue. Some of the crystals were separated and recrystallized from water. They were phenolsulphonamide, M.P. 170° (not depressed by authentic material, M.P. 176°) and gave the weak purple colour with $FeCl_3$ characteristic of the sulphonamide. The rest of the residue was dissolved in Na_2CO_3 solution and acetic anhydride added with cooling. After a short while, p-*acetoxybenzenesulphonamide* separated and was recrystallized (needles, M.P. 158°)[1] from 50 % aqueous alcohol. Since this acetyl derivative has not been previously described an authentic specimen was prepared. It formed long needles from 50 % aqueous alcohol, M.P. 159°, not depressed by the compound obtained through hydrolysis of the barium salt. (Found: N, 6·7 %. $C_8H_9O_4NS$ requires N, 6·5 %.) Another sample of the barium salt was hydrolysed and the crystalline residue obtained by ether extraction was benzoylated with benzoyl chloride and NaOH. A gum was obtained which, on crystallization from spirit, gave *p*-benzoyloxybenzenesulphonamide, M.P. 237°, as white plates. It did not depress the M.P. of an authentic sample, M.P. 238°. (Schreinemakers [1897] gives M.P. 234–236°.)

Methylation of the barium salt. The barium salt (5 g.) was dissolved in 50 ml. water and acetone was added until a slight permanent precipitate was obtained. The solution was then methylated at 35° with 25 ml. methyl sulphate and 60 ml. 30 % NaOH added dropwise with stirring. The stirring was continued for 5 hr. with occasional additions of acetone and then at 70° to destroy excess methyl sulphate and remove acetone. The solution was now cooled in ice, neutralized with $5N$ H_2SO_4 and made slightly acid to Congo red. Methylated spirit was added to precipitate Na_2SO_4 which was filtered under suction and thoroughly washed with spirit. The filtrate and washings were made slightly alkaline with dilute NaOH and concentrated to a thin syrup. The latter was now taken up in a small amount of $5N$ H_2SO_4 and the sticky precipitate formed was exhaustively extracted with chloroform. The chloroform solution was washed with water, dried over anhydrous Na_2SO_4 and then concentrated to an acid syrup (2·76 g.) which failed to crystallize. The syrup was now given three treatments with methyl iodide and silver oxide, i.e. until a constant methoxyl value was obtained. A yellow viscous syrup (2·2 g.) was obtained which could not be crystallized. This is presumably the *methyl ester* of 2:3:4-*trimethyl* p-*sulphondimethylamidophenylglucuronide*. It showed $[\alpha]_D^{21°} - 51·3°$ ($c = 1$, in $CHCl_3$). (Found: OMe, 29·4 %. $C_{18}H_{27}O_9NS$ requires OMe, 28·6 %.)

2:3:4-Trimethyl p-*sulphondimethylamidophenylglucuronamide.* The above methyl ester (0·5 g.) was dissolved in 5 ml. dry methyl alcohol and the solution saturated with dry ammonia at 0°. After 20 hr. in a desiccator at room temp. the solvent was evaporated and the residue crystallized on the addition of ether.

[1] Melting points are uncorrected.

The *amide* was recrystallized from acetone-ether. It formed long needles, M.P. 154–5°, slightly soluble in ether, but soluble in warm water, alcohol and acetone (yield 90 % theory). It showed $[\alpha]_D^{21°} -42{\cdot}3°$ ($c=0{\cdot}94$ in abs. alcohol) and $[\alpha]_D^{20°} -52{\cdot}2°$ ($c=1$, in water). (Found: C, 48·6; H, 6·1; N, 7·0; OMe, 23·1. $C_{17}H_{26}O_8N_2S$ requires C, 48·8; H, 6·2; N, 6·7; OMe, 22·25 %.)

Experiments with the oxidation product of phenolsulphonamide

The alcoholic filtrates and washings from the preparation of the barium salt were concentrated *in vacuo* to a syrup. This syrup was dissolved in absolute alcohol, leaving behind a quantity of the barium salt which had escaped precipitation in the original preparation. Three concentrations and solutions in alcohol were necessary to remove all the barium salt; finally, a syrup completely soluble in alcohol was obtained. The amount of this syrup was 1–1·5 g. from six rabbits fed with 2 g. each of phenolsulphonamide. The syrup was soluble in water and its solution did not reduce Fehling's solution, but reduced ammoniacal silver nitrate vigorously on warming. It gave the typical catechol $FeCl_3$ reaction (green, blue-green, purple, red on increasing the alkalinity) and gave an intense naphthoresorcinol reaction for glucuronic acid. One sample was methylated with methyl sulphate and alkali and the product extracted with ether. The ethereal solution was evaporated to a thick liquid and distilled. A brownish distillate was obtained, but no veratrole (dimethyl catechol) could be detected in it and the nature of the distillate was not discovered. Another methylated sample was subjected to treatment with various solvents in an attempt to crystallize out veratrole-4-sulphonamide, an authentic sample [Paul, 1906] of which had been prepared for comparison. No veratrolesulphonamide was obtained. Another sample of the syrup (1·5 g.) was hydrolysed by boiling under reflux with 20 ml. of 2*N* HCl. The hydrolysate was then extracted with ether in a continuous extractor. On evaporation of the extract a brownish crystalline residue was obtained which gave an intense green colour with $FeCl_3$. Treatment of this residue with Na_2CO_3 and acetic anhydride produced *p*-acetoxybenzenesulphonamide. The isolation of this compound, together with the fact that the hydrolysate was very strongly reducing, indicated that the syrup was mainly *p*-sulphonamidophenylglucuronide, containing a small amount of the catechol derivative. The isolation of the latter is now being tackled by other methods in conjunction with other work of a similar nature in this Laboratory.

Summary

The fate of *p*-hydroxybenzenesulphonamide (phenol-*p*-sulphonamide) in the rabbit has been studied. At a dose level of 0·75 g./kg. about 50 % of the dose is excreted conjugated with glucuronic acid, about 30 % with sulphuric acid and a small amount is oxidized to a catechol derivative, probably catecholsulphonamide. No free phenolsulphonamide was excreted.

A study was also made of the ethereal sulphate conjugation of phenolsulphonamide at various dose levels and it was found that the percentage conjugated fell with increasing dose.

The conjugated glucuronide of phenolsulphonamide was isolated as a barium salt and characterized by means of the crystalline amide of the 2:3:4-trimethyl-*p*-sulphondimethylamidophenyl-*β*-*d*-glucuronide.

It has been shown that phenol-*p*-sulphonic acid, 1-hydroxy-2-amino-, 1-amino-2-hydroxy- and 1-amino-3-hydroxy-benzene-4-sulphonic acids do not conjugate with glucuronic and sulphuric acids in the rabbit and are probably excreted unchanged.

REFERENCES

Baumann & Herter (1877–8). *Hoppe-Seyl. Z.* **1**, 244.
—— & Preusse (1879). *Hoppe-Seyl. Z.* **3**, 156.
Buttle, Gray & Stephenson (1936). *Lancet*, **1**, 1289.
Flaschenträger, Bernhard, Löwenberg & Schläpfer (1934). *Hoppe-Seyl. Z*, **225**, 157.
Friedländer (1896). *Fortschr. Teerfarb.* **3**, 59.
—— (1911). *Fortschr. Teerfarb.* **9**, 148.
Horn (1936). *Hoppe-Seyl. Z.* **238**, 84.
Jaffe & Hilbert (1888). *Hoppe-Seyl. Z.* **12**, 295.
Kleine (1896–7). *Hoppe-Seyl. Z.* **22**, 327.
Paul (1906). *Ber. dtsch. chem. Ges.* **39**, 2773.
Pryde & Williams (1933). *Biochem. J.* **27**, 1197.
—— —— (1934). *Biochem. J.* **28**, 133.
Schreinemakers (1897). *Rec. Trav. chim. Pays-Bas*, **16**, 423.
Scudi (1940). *Science*, **91**, 486.
Shelswell & Williams (1940). *Biochem. J.* **34**, 528.
Thorpe & Williams (1940). *Nature, Lond.*, **146**, 686.
—— —— (1941). *Biochem. J.* **35**, 61.
—— —— & Shelswell (1941). *Biochem. J.* **35**, 52.
Wiley (1938). *J. biol. Chem.* **124**, 627.
Williams (1938). *Biochem. J.* **32**, 878.

Note added 27 May 1941. The catechol derivative mentioned above has now been identified as catechol-4-sulphonamide, since suitable treatment, including methylation, of ether extracts of the hydrolysed urine gave crystalline *veratrole-4-sulphondimethylamide*, M.P. 105°, identical with a synthetic sample. Synthetic *catechol-4-sulphonamide* has also been prepared and found to be a non-crystalline resin, although *catechol-4-sulphonanilide* is crystalline, M.P. 221°. A description of this work will be reported when completed.

59. THE HYDROLYTIC DEGRADATION OF THE ANTIGENIC COMPLEX OF *BACT. TYPHOSUM* Ty2

By GEORGE GROSVENOR FREEMAN[1] and THOMAS HEMINGWAY ANDERSON

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London

(*Received 13 March 1941*)

The antigens of a number of Gram-negative micro-organisms have been isolated in a relatively pure condition and apparently unchanged in their specific immunological properties by various methods in the work of Boivin *et al.* [1933; 1934; 1935; 1937], Raistrick & Topley [1934], Topley *et al.* [1937], Morgan [1937], Morgan & Partridge [1940], and Miles & Pirie [1939, 1, 2, 3]. By use of the trichloroacetic acid method of extraction of Boivin *et al.* and the tryptic digestion method of Raistrick & Topley, Freeman *et al.* [1940] obtained the antigens of *Bact. typhosum* and *Bact. typhi-murium* and showed that by growth of the organisms in a synthetic medium various sources of contamination were eliminated.

The present communication describes a more detailed investigation of the chemical nature of the antigenic fractions obtained from *Bact. typhosum* Ty2 by the above methods.

The Ty2 strain of *Bact. typhosum* and its isolated antigen react with both O and Vi antisera; this is regarded by some authors as being due to the presence of two chemically and immunologically distinct entities, the O and Vi antigens. Boivin & Mesrobeanu [1938, 2] claim to have separated these components by precipitating the Vi antigen with uranyl acetate but attempts to repeat this work by the present authors have so far proved unsuccessful. Malek [1939] was also unable to separate the O and Vi component antigens by means of uranyl acetate. Meyer [1938] has, however, stated that the O and Vi antigens of an O+Vi strain of *Bact. typhosum* are distinct molecular entities which can be separated by their precipitation reactions towards specific antisera. Thus, whilst in the investigation to be described the antigen of *Bact. typhosum* Ty2 has been regarded as an individual molecule, the possibility of its consisting of component O and Vi antigenic molecules must not be overlooked.

Experimental

The bulk of the starting material in this investigation was prepared from the dried cells of *Bact. typhosum* Ty2, grown on a synthetic medium, by tryptic digestion as described by Freeman *et al.* [1940].

Properties of the antigenic complex

The tryptic digest antigen of *Bact. typhosum* Ty2 dissolves readily in water giving opalescent solutions even when the concentration of antigen is as low as

[1] Grocers' Company Scholar for Medical Research.

0·4 %. The bulk of the substance remains in solution after 30 min. centrifuging at 15,000 r.p.m. The antigenic complex also dissolves readily in formamide and formic acid giving solutions which are practically free from opalescence, but it is insoluble in glacial acetic acid, dioxan and in concentrated aqueous solutions of phenol. Qualitative tests have been made on 0·5 ml. quantities of a 0·4 % aqueous solution of the antigen; the test solutions were centrifuged at about 2000 r.p.m. for 15 min. and 0·5 ml. of the opalescent supernatant solution was mixed with 0·5 ml. of the reagent. Acid potassium permanganate was slowly reduced by the antigenic solution in the cold and more rapidly on warming. Addition of uranyl acetate solution produced an immediate flocculent precipitate, whilst phosphotungstic acid in 5 % H_2SO_4 gave a precipitate on standing and neutral lead acetate an increased opalescence on standing. No precipitates were obtained on addition of Esbach's reagent, 10 % trichloroacetic acid solution, salicylsulphonic acid or tannic acid. Tyrosine was present as indicated by positive Millon's, xanthoproteic, α-nitroso-β-naphthol and Ehrlich's diazo reactions. The Adamkiewicz and aldehyde reactions and Ehrlich's *p*-dimethylaminobenzaldehyde test gave negative results showing the absence of tryptophan. The sodium fusion test and boiling of the antigen with strong NaOH and addition of lead acetate proved the absence of sulphur. A positive Sakaguchi reaction for arginine was obtained. The antigenic solution also gave positive biuret and ninhydrin reactions, indicating the presence of peptide linkages and amino-groups respectively. The substance gave a very strong Molisch's reaction but did not reduce Fehling's solution. Pentose residues (furfuraldehyde, Tollen's phloroglucinol and Bial's tests), ketose (Seliwanoff's and Foulger's tests) and glucuronic acid residues (Tollen's naphthoresorcinol reaction) were found to be absent. The solution gave no iodine reaction.

The nitrogen content and reducing sugar value of the antigenic preparations varied within narrow limits from batch to batch and a typical sample gave: N, 7·2 %; reducing sugar value, 32 %; $[\alpha]_{5461} = +104°$ (in aqueous solution, $c=1$), $[\alpha]_D^{17°} = +69°$ (in formamide solution, $c=1$), and $[\alpha]_D^{15°} = +68°$ (in formic acid solution, $c=1$); non-volatile ash, 12·2 %; organic P, 3·0 %; inorganic P, 1·2 % (N/organic P ratio, 5·0). Determination of amino-N by Van Slyke's method showed that 0·6 % of N was present as amino-groups.

The tryptic digest antigen was toxic for mice, having an average lethal dose of 0·3 mg. [Freeman *et al.* 1940]. Immunizing experiments similar to those described by Topley *et al.* [1937] have been carried out on practically every batch of antigen, and the results have shown that the substance possesses a high degree of immunological activity comparable with that of the original dried bacterial cells.

Acetic acid hydrolysis of the antigenic complex

The tryptic digest antigen was dispersed by shaking with 0·1 *N* acetic acid to give a 1 % solution, which was centrifuged at 15,000 r.p.m. for 2–3 hr. to remove undissolved material ('undissolved residue'). The supernatant solution which was slightly opalescent was hydrolysed at 100° on the boiling water bath for 12 hr. On heating, the solution first clarified a little (30 min.), but after 2 hr. it became very opalescent and an insoluble substance began to flocculate out. After about 8 hr. heating the flocculation appeared to be complete and there was no further deposition of precipitate and no change of optical rotation during the last 2 hr. The insoluble material ('hydrolysis precipitate') was centrifuged off, washed with a little water and dried *in vacuo* over phosphorus pentoxide.

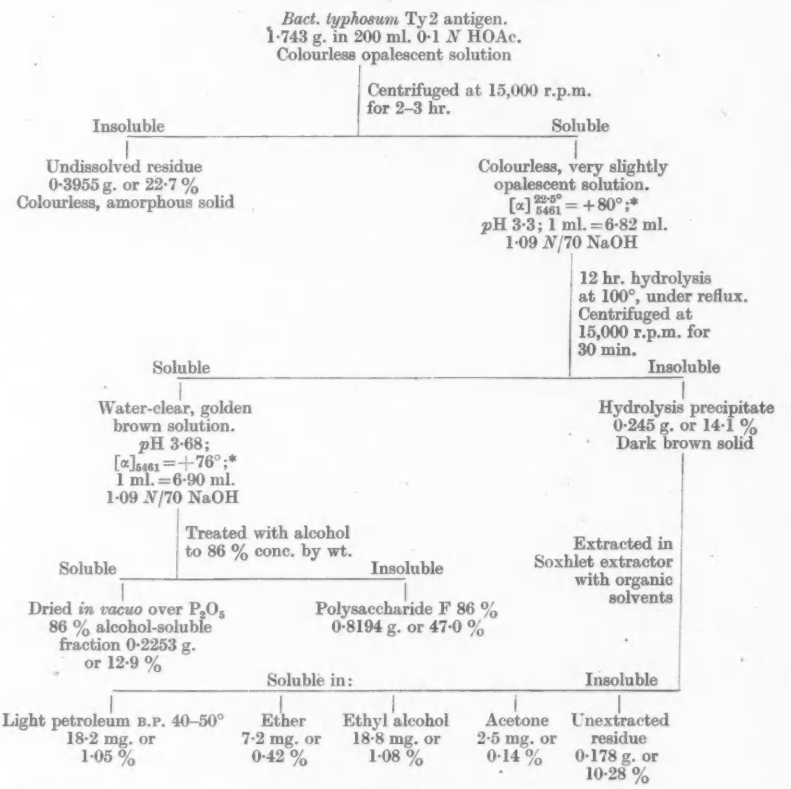
The dried hydrolysis precipitate was then exhaustively extracted in a Soxhlet extractor, successively with:

(1) Light petroleum, B.P. 40–50°,
(2) Pure anhydrous ether,
(3) Absolute ethyl alcohol, and
(4) Acetone.

The solvents were evaporated from the extracted lipid components, which were stored *in vacuo*. The remainder ('unextracted residue') was dried *in vacuo* over phosphorus pentoxide.

The acetic acid supernatant solution, containing the soluble hydrolysis products, was water-clear and golden brown in colour; it was treated with sufficient absolute alcohol to bring the alcoholic concentration to 86%, by weight, giving a precipitate rich in polysaccharide ('Polysaccharide F 86 %'). This

Table 1. *0·1N acetic acid hydrolysis of* Bact. typhosum *Ty2 'tryptic digest antigen'*



* Uncorrected for ash.

Total recovery of fractions = 96 %.

fraction was washed twice with absolute alcohol, twice with ether and dried *in vacuo* over phosphorus pentoxide.

The alcoholic supernatant solution and washings of polysaccharide F 86 % were evaporated to dryness *in vacuo*, giving a brown-coloured sticky residue: '86 % alcohol-soluble fraction'.

The fractionation is summarized in Table 1. The fractions thus isolated have been submitted to qualitative and quantitative examination; Kjeldahl N, non-volatile ash, reducing sugar value, specific optical rotation, total and inorganic P, amino-N and acidity were determined. The N, ash, reducing sugar value and specific rotation determinations were carried out as described by Freeman *et al.* [1940]. Total P determinations were made on 0·5–1·0 mg. quantities of substance by Stewart & Hendry's [1935] modification of the Fiske & Subbarow [1925] method. Inorganic P determinations were made on 2–5 mg. quantities of substance by the Fiske & Subbarow method. Organic P has been taken as the difference between total and inorganic P, and equivalent N/organic P ratios have been calculated on this basis. The amino-N content of the soluble fractions has been determined on 10 mg. samples by the Van Slyke method. Acidity determinations were made by titrating 5–10 mg. quantities dissolved in a few ml. water with 0·014 *N* NaOH using phenolphthalein as indicator. Except where otherwise stated analytical results are quoted on an ash-free basis. The results obtained for the fractions isolated by partial acetic acid hydrolysis of the antigenic material are summarized in Table 2.

Table 2. *Analytical data of fractions isolated from* Bact. typhosum *Ty2 antigen* (1·74 g.)

	Un-dissolved residue	Un-extracted residue	Total lipid	Poly-saccharide F 86 %	86 % alcohol-soluble fraction
Weight (g.)	0·395	0·178	0·047	0·819	0·225
Yield (%)	22·7	10·3	2·7	47·0	12·9
N (%)	8·9	6·0	0·6	3·6	13·4
Non-volatile ash (%)	7·4	5·0	—	20·2	5·4
Reducing sugar value (%)	28	10	—	55	19
$[\alpha]_{5461}$	—	—	—	+142°	+7°
Inorganic P (%)	—	—	—	3·0*	0·2*
Organic P (%)	4·1	2·2	0·5	1·6	0·6
N/org. P ratio	4·7	6·0	2·6	4·8	48
Amino-N (%)	—	—	—	0·5	3·0
Acidity ml. *N* NaOH/g.	—	—	—	0·55*	1·38*
	* Uncorrected for ash.				
% Yield on total dissolved matter	—	13·3	3·5	60·8	16·7

Further samples of antigenic material were hydrolysed under slightly different conditions with similar results. Soru & Combiesco [1940] report fission of the *Bact. typhosum* O-antigen, prepared by trichloroacetic acid extraction of the organisms, by hydrolysis with 0·04 *N* acetic acid for 45–60 min. at 100°. Some of our hydrolyses were made with 0·1 *N* acetic acid for 2 hr., and in one experiment a trichloroacetic acid extract of the bacteria, 'Boivin antigen F 68 %', was hydrolysed with 0·04 *N* acetic acid for 60 min. at 100°. In these cases in which a shorter period of hydrolysis was used the flocculation of the hydrolysis precipitate was incomplete and this fraction could not be completely precipitated on centrifuging. Addition of alcohol to about 30 % concentration, by weight, was required to complete the flocculation of the hydrolysis precipitate. (The polysaccharide component is completely precipitated between the limits

Table 3. *Hydrolysis of further batches of* Bact. typhosum *Ty2 antigen with dilute acetic acid*

Conditions of hydrolysis, etc.		Hydrolysis precipitate		Polysaccharide F 86 %	86 % alcohol-soluble fraction
1. 3·644 g. tryptic digest antigen dissolved in 335 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	0·493		1·937	0·498
	% of antigen ...	13·6		53·2	13·7
	Properties ...	Main fraction after solution in *N* NaOH and repptn. by HCl and extraction with organic solvents gave: N 8·2 %, R.V. 10 %, ash 2·0 %, P (total) 2·4 %		After two fractionations between 42 % and 85 % conc. of alcohol, by weight, gave: N 2·2 %, R.V. 68 %, ash 6·9 %	P (total) 1·9 % Ash 9·0 % R.V. 19 % N 14·3 % Amino-N 1·8 %
2. 5·07 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 12 hr.	Yield g. ...	1·122		2·602	1·157
	% of antigen ...	22·8		53·1	23·6
3. 4·54 g. tryptic digest antigen dissolved in 500 ml. 0·1 *N* acetic acid and hydrolysed at 100° for 2 hr.	Yield g. ...	1·1576		2·721	0·2926
	% of antigen ...	25·5		59·9	6·4
4. 0·598 g. 'Boivin antigen F 68 %' dissolved in 112·5 ml. 0·04 *N* acetic acid and hydrolysed at 100° for 60 min.		Ppt. on centrifuging	Alcoholic ppt. F 50 %	(F 50–85 %)	
	Yield g. ...	0·0156	0·3092	0·1828	—
	% of antigen ...	2·6	51·7	30·6	—
	Properties ...	N 6·0 %	N 6·4 % Ash 14·8 %	R.V. 64 % Ash 8·1 % N 2·57 % P (total) 1·8 %	—

of 40–85 % concentration of alcohol, by weight, but does not precipitate to any appreciable extent at 30 % alcoholic concentration.) These experiments are summarized in Table 3.

The undissolved residue which may be separated from solutions of the antigenic material by high-speed centrifuging, as in the original hydrolysis experiment, is to be regarded as an insoluble or 'denatured' modification of the antigen, which tends to pass into an insoluble form on drying and storage. This fraction, therefore, has not been removed in the later experiments but submitted to hydrolysis with the bulk of the antigenic material.

The experiments 1–3, summarized in Table 3, confirm the results of the original hydrolysis experiment (Tables 1 and 2); when the time of hydrolysis is shortened to 2 hr. (exp. 3) the separation into soluble and insoluble fractions was much less definite and the hydrolysis precipitate was not completely flocculated until an alcoholic concentration of 32 % (by weight) had been reached. 6 % of insoluble substance was removed by centrifuging at 3000 r.p.m. on an angle centrifuge for 4 hr., leaving a very opalescent supernatant solution, from which the remaining 19·5 % of insoluble matter was precipitated by an alcoholic concentration of 32 % (by weight) leaving a water-clear supernatant solution. In this experiment the 86 % alcohol-soluble fraction was also much smaller in amount. In exp. 4, in which a trichloroacetic acid extract of *Bact. typhosum* Ty2 'Boivin antigen F 68 %') was hydrolysed under the conditions employed by Soru & Combiesco [1940], there was an even greater difficulty in flocculating the insoluble fraction. Only 2·6 % could be precipitated by centrifuging (3000 r.p.m. on an angle centrifuge for 3 hr.), and alcohol to 50 % concentration (by weight) had to be added in order to complete the flocculation of the very opalescent supernatant solution. This produced 52 % of substance, which was probably incompletely hydrolysed. A crude polysaccharide, alcoholic fraction F 50–85 %, was obtained which was very similar in properties to the crude polysaccharide obtained on hydrolysis of the tryptic digest antigen.

The mean of the results obtained in exps. 1–3 and in the original hydrolysis experiment (Table 2) point to the conclusion that on mild hydrolysis the antigen of *Bact. typhosum* Ty2 yields about 57 % of crude polysaccharide, 18 % of alcohol-soluble fraction and 20 % of hydrolysis precipitate, of which about 4 % (in terms of the original weight of antigen) is of a lipid nature. These four constituents will now be considered in fuller detail.

The polysaccharide component of the antigenic material

The crude polysaccharide was a water-soluble, almost colourless amorphous substance, which corresponded to about 57 % of the antigenic complex. As first precipitated it contained 3·6 % N, 1·6 % organic P and a considerable amount of ash. On hydrolysis with 2*N* HCl for 2 hr. in a sealed tube 55 % of reducing sugars were liberated (Hagedorn & Jensen). At this stage the polysaccharide had a small titratable acidity corresponding to 0·5 ml. *N* NaOH/g. and had a high specific rotation of about $[\alpha]_{5461} + 140°$. The polysaccharide dissolved readily in water, giving solutions which were water-clear. Solutions of the crude material failed to give a positive Millon's reaction or α-nitroso-β-naphthol test for tyrosine; the biuret reaction was also negative. Weakly positive ninhydrin tests and the Sakaguchi reaction for arginine indicated that small amounts of amino-acid residues remained as impurities in the polysaccharide. This was confirmed by the appearance of a trace of precipitate on addition of phosphotungstic acid in 5 % H_2SO_4 and of an opalescence on addition of neutral lead acetate. The polysaccharide gave a very strongly positive Molisch's reaction. It gave no coloration

with iodine and failed to reduce Fehling's solution. There was no evolution of furfuraldehyde on heating with 12 % HCl. Bial's reaction and Tollen's phloroglucinol test for pentoses were also negative as were the Seliwanoff test and Foulger's test for ketoses. Tollen's naphthoresorcinol test indicated the absence of uronic acid residues.

The purification of the polysaccharide by means of alcoholic and acetic acid fractionations or by preparation of the triacetyl derivative and subsequent deacetylation will be described in a later communication. An identical polysaccharide was obtained by treatment of the dried bacterial cells with dilute acetic acid at 100°. After purification the polysaccharide was obtained free from N and contained only traces of P (0·1–0·2 %). The purified substance had a reducing value of 1·1 % before hydrolysis, and on hydrolysis with 2*N* HCl for 2 hr. at 100° in a sealed tube gave 83 % of reducing sugars (Hagedorn & Jensen). It was highly dextrorotatory, $[\alpha]_D = +114°$ and $[\alpha]_{5461} = +140°$. Sulphur was absent and the ash content had been reduced to 1–2 %. On hydrolysis the polysaccharide yielded 3 % of volatile fatty acid (calculated as acetic acid), indicating the presence of acetyl residues in the molecule. (Found: C, 44·41; H, 7·12 %. $C_6H_{10}O_5$ requires C, 44·41; H, 6·22 %.)

The above-mentioned qualitative tests on the crude material indicate the absence of ketose, pentose and uronic acid residues from the polysaccharide molecule and repetition of the tests on the purified substance gave the same results, showing that such residues are either entirely absent or present only in very small amounts. On hydrolysis of the polysaccharide, derivatives of *d*-mannose, *d*-galactose and *d*-glucose have been isolated; these hexoses constitute the bulk of the polysaccharide molecule.

Our colleague, Dr A. Felix, has shown that solutions of the polysaccharide precipitate with 'pure' *Bact. typhosum* O-antiserum to a dilution of 1 in 15,000,000 of the polysaccharide, but not with 'pure' Vi-antiserum. The polysaccharide represents the O-hapten of the bacterium.

The polypeptide component of the antigenic material ('unextracted residue')

The fraction which is insoluble in water and organic solvents ('unextracted residue') constitutes about 16 % of the antigenic complex. In its crude state it is obtained as a brownish coloured solid which dissolves almost completely in 2*N* NaOH and is reprecipitated on acidification. Identification of constituent residues in the unhydrolysed substance is difficult owing to its insolubility. Although the ninhydrin reaction is negative, the presence of tyrosine and arginine is evident from positive xanthoproteic, Millon's and α-nitroso-β-naphthol tests, and the Sakaguchi reaction respectively. After partial hydrolysis with mineral acid a positive biuret reaction is obtained.

Fractionation of the hydrolysis precipitate. The dark greyish-brown hydrolysis precipitate obtained in exp. 1 (Table 3), and unextracted with organic solvents, was suspended in water and NaOH was added to *N* concentration. The bulk of the precipitate dissolved in the alkali to give a dark brown solution, leaving a small amount of undissolved residue which was removed by centrifuging. This undissolved residue was suspended in a few ml. of 2*N* NaOH when it showed anisotropy of flow and remained undissolved. It was recovered by acidification as a greyish-white precipitate. This was dried *in vacuo* over P_2O_5 and yielded 0·035 g. of 'hydrolysis precipitate (fraction insoluble in NaOH and HCl)'.

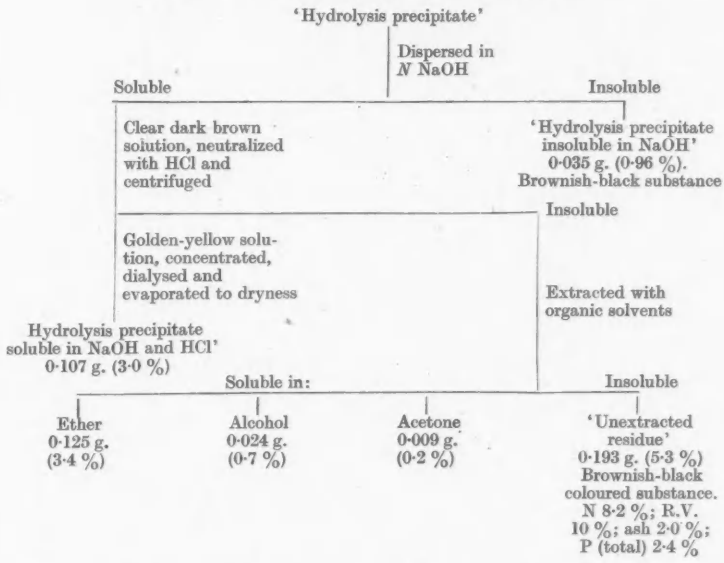
The clear brown alkaline solution containing the bulk of the hydrolysis precipitate was made slightly acid with HCl when a buff-coloured precipitate

of the acidic hydrolysis precipitate was obtained. The solid was centrifuged, taken up in a small quantity of 0·1 *N* NaOH in which it dissolved readily, and reprecipitated by neutralization with HCl; the precipitate was finally washed free from chlorides with distilled water.

The golden-yellow acid supernatant solution obtained on reprecipitation of the hydrolysis precipitate was neutralized and concentrated *in vacuo*. The concentrated opalescent suspension was dialysed in a collodion sac against frequent changes of distilled water for 24 hr. to remove sodium chloride, etc. and the contents of the sac were evaporated to dryness *in vacuo* giving 0·107 g. of a brown substance: 'Hydrolysis precipitate (fraction soluble in NaOH and HCl)'.

The bulk of the hydrolysis precipitate (i.e. soluble in NaOH and reprecipitated by HCl) was suspended in 20 ml. water and extracted with ether in a continuous liquid extraction apparatus for 40 hr. The ethereal solution on evaporation yielded 0·125 g. of a light brown waxy substance. The unextracted brown hydrolysis precipitate was centrifuged down and dried *in vacuo* over P_2O_5 yielding 0·290 g. of a brownish-black substance. This was extracted in a Soxhlet extractor with absolute ethyl alcohol for 24 hr., and 0·0238 g. of brown fatty material was recovered from the alcoholic solution. The unextracted residue was then extracted with acetone for 24 hr. in a Soxhlet extractor and 0·009 g. of lipid material was recovered from the acetone extract. The final unextracted residue consisted of 0·193 g. of a dark brown amorphous substance. The above fractionation is summarized in Table 4.

Table 4. *Fractionation of 'hydrolysis precipitate'*


'Hydrolysis precipitate'
Dispersed in N NaOH
Soluble
Insoluble
Clear dark brown solution, neutralized with HCl and centrifuged
'Hydrolysis precipitate insoluble in NaOH' 0·035 g. (0·96 %). Brownish-black substance
Insoluble
Golden-yellow solution, concentrated, dialysed and evaporated to dryness
Extracted with organic solvents
Hydrolysis precipitate soluble in NaOH and HCl' 0·107 g. (3·0 %)
Soluble in:
Insoluble
Ether 0·125 g. (3·4 %)
Alcohol 0·024 g. (0·7 %)
Acetone 0·009 g. (0·2 %)
'Unextracted residue' 0·193 g. (5·3 %) Brownish-black coloured substance. N 8·2 %; R.V. 10 %; ash 2·0 %; P (total) 2·4 %

The purified fraction obtained in the above fractionation contains N 8·2 %, P (total) 2·4 % and has a reducing value on hydrolysis with 2 *N* HCl for 2 hr. in a sealed tube at 100° of 10 % apparent glucose. The substance is acidic, being

readily soluble in dilute alkalis in the cold, but insoluble in water and dilute acids; it is so resistant to acid hydrolysis that it is only partially soluble on treatment with 2*N* HCl in a sealed tube for 2 hr. at 100°. The partial hydrolysate thus obtained gives an intense yellowish-red coloration with diazobenzene-sulphonic acid in alkaline solution (Ehrlich's diazo-reaction) and a positive α-nitroso-β-naphthol reaction indicating the presence of tyrosine. The ninhydrin reaction is also strongly positive at this stage.

Tryptic digestion of the 'unextracted residue'. The action of trypsin on the polypeptide-like fraction ('unextracted residue') has been investigated and the liberation of amino-acids during tryptic digestion followed by means of the Van Slyke method. 0·075 g. of the 'unextracted residue', prepared as described in Table 4, was freshly precipitated by acidifying an alkaline solution of the substance and then suspended in 15 ml. of borate buffer at *p*H 8·46. A brownish-coloured opalescent suspension was obtained. 0·003 g. commercial trypsin (Fairchild Bros. and Foster, New York) was added and the digest incubated at 37° for 14 days at *p*H 8·2–8·5, a further 0·003 g. trypsin being added after 2 days' digestion. 1 ml. portions of the digest were withdrawn at intervals and the amino-N estimated by the Van Slyke method. A blank determination was also made on a sample of trypsin which itself contained a small amount of amino-N. After 2 days' digestion only 7·3 % of the total N had been liberated as amino-N and 13·4 % after 14 days. The substance was therefore only slowly and very incompletely attacked by trypsin; its resistance to tryptic digestion is evident from the fact that after 14 days' exposure to the enzyme under these conditions an insoluble substance similar to the undigested compound was precipitated on acidification.

Acid hydrolysis of the 'unextracted residue'. Morgan & Partridge [1940] found that the polypeptide component of the *B. dysenteriae* (Shiga) antigenic complex was readily hydrolysed by 10*N* formic acid containing 3·6 % HCl. It has already been pointed out that the polypeptide-like fraction from the *Bact. typhosum* Ty 2 antigenic complex was not completely dissolved by treatment with 2*N* HCl for 2 hr. in a sealed tube at 100°. 0·008 g. quantities of the 'unextracted residue' were treated with 1 ml. 10*N* formic acid containing 3·6 % HCl and hydrolysed in sealed tubes at 100° for various lengths of time. At the end of the periods of hydrolysis the acid hydrolysates were exactly neutralized with NaOH and the amino-N determined by the Van Slyke method. The substance did not dissolve completely in the acid mixture even after 10 hr. hydrolysis. The results are summarized in Table 5.

Table 5. *Rate of increase of amino-N during acid hydrolysis of the 'unextracted residue' of the* Bact. typhosum *Ty 2 antigenic complex*

Weight of sample mg.	Time hr.	Amino-N per ml. mg.	Total N per ml. mg.	Amino-N as % of total N
—	0	Substance insoluble	—	—
8·60	0·5	0·081	0·686	11·8
8·50	2	0·209	0·678	30·8
8·50	3	0·254	0·678	37·4
8·80	5	0·317	0·702	45·2
8·85	6	0·317	0·706	44·9
8·65	10	0·332	0·690	48·1

The results in Table 5 show that during acid hydrolysis the amino-N of the fraction of the antigenic complex (insoluble in acids and organic solvents)

increases to 48 % of the total N after 10 hr. hydrolysis, and that at this stage the liberation of amino-acids has reached a maximum. The insoluble nitrogenous component of the antigen contains tyrosine and arginine, it liberates 13·4 % of its total nitrogen as amino-groups on prolonged tryptic digestion, and 48 % of amino-N on acid hydrolysis and must therefore be regarded as a polypeptide.

The 86 % alcohol-soluble fraction of the antigenic complex

This fraction contains N 13·4 %, P (inorganic 0·2 %, organic 0·6 %), and has a small reducing power of 13 % (apparent glucose) which increases to 18 % on hydrolysis with 2*N* HCl in a sealed tube for 2 hr. at 100°. Of the total N, 3·0 % is in the form of amino-groups (Van Slyke). In aqueous solution the substance has a small dextrorotation, $[\alpha]_{5461}^{20°}+7°$ ($c=1$). The substance dissolves readily in cold water, leaving a trace of insoluble residue; a 0·4 % solution was subjected to various qualitative tests. Acid $KMnO_4$ solution was reduced in the cold. Positive biuret and ninhydrin reactions showed the presence of peptide linkages and amino-groups respectively, and that these were due to the presence of tyrosine residues was indicated by positive xanthoproteic and Millon's reactions. The presence of arginine was indicated by the Sakaguchi reaction, but the aldehyde test for tryptophan gave negative results. An immediate bulky precipitate was obtained, with phosphotungstic acid in 5 % H_2SO_4 and a precipitate on standing with neutral lead acetate, but no precipitate was obtained with 10 % trichloroacetic acid or tannic acid solution. A positive Molisch's reaction was obtained, but no furfuraldehyde was obtained on heating a sample with 12 % HCl, and Foulger's test for ketoses was also negative.

Whilst the alcohol-soluble fraction may be expected to contain any low-molecular substances liberated by acetic acid hydrolysis, e.g. sugars, amino-acids, etc., and the above quantitative and qualitative examinations would appear to confirm the presence of small amounts of these substances, there remains nevertheless about 10 % of N unaccounted for by amino-N. The presence of arginine and tyrosine and the precipitability of the fraction with phosphotungstic acid and neutral lead acetate are not inconsistent with the view that a water-soluble polypeptide may be present. Attempts to break down the substance by tryptic digestion and hydrolysis with mineral acids to produce free amino-acids have so far been unsuccessful.

Action of trypsin on alcohol-soluble fraction. 0·15 g. of the dried substance was dissolved in 15 ml. of a veronal-HCl buffer (*p*H 8·5), 0·003 g. trypsin (Fairchild Bros. and Foster, New York) added and the mixture incubated at 37° and *p*H 8·2–8·5; a further 0·003 g. trypsin was added after 2 days' digestion. Before addition of trypsin 7·5 % of the total N was present as amino-N (Van Slyke); this increased to 9·7 % after 2 days' digestion and 11·3 % after 5 days' digestion. It is evident, therefore, that the substance was not degraded to any significant extent by trypsin.

Acid hydrolysis of alcohol-soluble fraction. Acid hydrolysis of the alcohol-soluble fraction was investigated under the same conditions as those employed for the hydrolysis of the insoluble polypeptide fraction. 10 mg. samples of the dried solid were heated at 100° in sealed tubes with 1 ml. quantities of 10*N* formic acid containing 3·6 % HCl. After 2 hr. heating 12·4 % of the total N was liberated as amino-groups and 16·4 % after 7 hr., as compared with 45 % after 7 hr. in the case of the water-insoluble polypeptide. The action of conc. HCl [cf. Mitchell & Hamilton, 1929] on the alcohol-soluble fraction was also investigated. 10 mg. quantities of the substance were heated at 100° in sealed tubes with 1 ml. concentrated HCl; after 6 hr. 18·0 % of the total N was

liberated as amino-N and 18·3 % after 8 hr. heating. It appears unlikely that the alcohol-soluble component of the antigen is a polypeptide and the nature of this fraction remains obscure; investigation of this problem is being continued.

The lipid components of the antigenic complex

Taken as a whole the products of the acetic acid hydrolysis of *Bact. typhosum* Ty2 antigenic complex, which are soluble in organic solvents (light petroleum, ether, ethyl alcohol and acetone) constitute a total of only 2·7 % of the original antigen (Table 1). (In another experiment under similar conditions 4·3 % of substance soluble in these solvents was obtained.) Although other workers have isolated much larger amounts of lipid components of bacterial antigens [Morgan & Partridge, 1940; Soru & Combiesco, 1940] our *Bact. typhosum* Ty2 antigen does not appear to liberate a significant quantity of lipid components on acetic acid hydrolysis.

The light petroleum- and ether-soluble fractions described in Table 1 were combined by dissolving in an ether-alcohol mixture and N and P determinations made, giving N 0·6 %, P 0·5 % and N/P ratio 2·6. 0·15 g. of the lipid fraction was dissolved in 20 ml. ether and extracted with aqueous Na_2CO_3, when the bulk of the lipid material passed into the alkaline aqueous solution. This latter fraction was recovered by acidification and extraction with ether. The ethereal solution was dried over anhydrous Na_2SO_4, filtered and evaporated to dryness, yielding 0·094 g. of a partially crystalline yellow-orange fatty substance. On analysis it gave N 1·2 %, P 1·0 %, N/P ratio 2·8. The material unextracted from ethereal solution by aqueous Na_2CO_3 was also recovered and found to be almost completely soluble in acetone. It appears, therefore, from a preliminary examination, that the lipid fraction consists predominantly of a mixture of fatty acids while a phospholipin may also be present in small amounts.

The action of trypsin on the tryptic digest antigen of Bact. typhosum *Ty2*

The antigens of Gram-negative organisms have generally been regarded as resistant to digestion by proteolytic enzymes [Boivin & Mesrobeanu, 1938, 1]. Morgan & Partridge [1940] have, however, been able to destroy the antigenicity of the *B. dysenteriae* (Shiga) antigen by prolonged treatment with relatively large quantities of trypsin. By tryptic digestion they degraded the complete antigen (a phospholipin-polysaccharide-polypeptide complex) into an antigenically inactive phospholipin-polysaccharide complex. It seemed unlikely that the *Bact. typhosum* Ty2 antigenic complex, which is isolated in an immunologically active and toxic condition by prolonged tryptic digestion of the bacterial cells, would be susceptible to attack by trypsin, but the further effect of relatively large quantities of the enzyme for a long period was investigated as follows. 0·206 g. of tryptic digest antigen was dissolved in 20 ml. veronal-HCl buffer (*p*H 8·5), and the solution treated with 0·008 g. trypsin (Fairchild Bros. and Foster, New York); the digest was incubated at 37° and *p*H 8·2–8·5. The course of the tryptic digestion was determined on 1 ml. samples of digest by the Van Slyke method for determination of amino-N. After 60 hr. digestion a further 0·03 g. trypsin was added and the experiment continued for 12 days in all. The results are summarized in Table 5.

Thus even when the antigen is submitted to prolonged attack by relatively large amounts of trypsin (approximately 20 times as much as that used in the tryptic digestion of the bacterial bodies) it undergoes an insignificant degradation

Table 5. *Rate of liberation of amino-N during tryptic digestion of* Bact. typhosum *Ty2 antigenic complex*

Time in hr.	Amino-N per ml. (mg.)	Total N per ml. (mg.)	Amino-N as % of total N
0	0·068	0·747	9·0
3	0·059	0·747	8·0
60	0·082	0·747	11·0
72	0·099	0·747	13·3
288	0·108	0·747	13·5

as indicated by liberation of only a further 4·5 % of the N as amino-groups. It is concluded that the tryptic digest antigen of *Bact. typhosum* Ty2 is stable towards further attack by this enzyme.

Discussion

Two main constituents of the antigenic complex of *Bact. typhosum* Ty2 have been isolated and investigated. These consist of a polysaccharide, which constitutes 50–60 % of the antigen and an acidic, water-insoluble polypeptide, constituting about 16 % of the complex. The polysaccharide, which has been obtained free from N and all but traces of P, gives on analysis C, 44·4; H, 7·1; acetyl, 3 %; and has been shown to consist of *d*-mannose, *d*-galactose and *d*-glucose units. An identical polysaccharide, $[\alpha]_D^{20°}+114°$, $[\alpha]_{5461}^{20°}+140°$, R.V. = 83 % apparent glucose, has been isolated directly from the dried bacterial cells by extraction with dilute acetic acid. Precipitation reactions with 'pure' O- and Vi-antisera have shown that the polysaccharide reacts specifically with O-antiserum but not with Vi-antiserum (in contrast to the antigenic complex from which it is derived), and is to be regarded as the O-specific hapten of *Bact. typhosum*. The polysaccharide probably represents, in a pure form, the soluble specific polysaccharide of *Bact. typhosum*, which White [1929] obtained, together with similar polysaccharides from other Salmonellas. Topley *et al.* [1937] also reported 'a highly dextrorotatory polysaccharide' on hydrolysis of virulent strains of *Bact. typhosum*. Earlier studies on crude bacterial polysaccharides have indicated that, as compared with the complete antigenic complex, they are relatively non-toxic and lacking in antigenic properties. Soru & Combiesco [1940] have isolated a polysaccharide on hydrolysis of the O-antigen of *Bact. typhosum*, with dilute acetic acid, which is probably identical with our polysaccharide though not yet pure. The polysaccharide of Soru & Combiesco is free from N, gives 72·4 % of reducing sugars on hydrolysis, is highly dextrorotatory ($[\alpha]+125°$) and contains 11·65 % of ash and 2·88 % of P (results, except that for P, calculated on an ash-free basis).

The polysaccharide hapten, which determines the specificity of the O-antigen of *Bact. typhosum*, has a parallel in the polysaccharide haptens of other organisms such as *B. dysenteriae* (Shiga), the specific polysaccharide of which, prepared by similar methods to those used in the present work, contains N (1·6 %), gives 97 % of reducing sugars on hydrolysis and is dextrorotatory ($[\alpha]_{5461}=+98°$). This polysaccharide is built up, in part, of *d*-galactose, *l*-rhamnose and *N*-acetylglucosamine units [Morgan, 1938; Jolles & Morgan, 1940].

The other well-characterized component of the *Bact. typhosum* Ty2 antigenic complex, the polypeptide-like component, probably plays a non-specific role in the complex antigen and is to be regarded as producing, in combination with the polysaccharide, a molecule of sufficient complexity to possess antigenic properties, the specific nature of which is dominated by the polysaccharide. The polypeptide component contains 8·2 % N and 2·4 % P; tyrosine and arginine

have been identified. The compound is fairly readily hydrolysed with 10*N* formic acid which contains 3·6 % HCl at 100° with liberation of 48 % of the N as free amino-groups (Van Slyke), but it is relatively very resistant to tryptic digestion. Soru & Combiesco [1940] obtained 21·7 % of an ether- and water-insoluble fraction from *Bact. typhosum* O-antigen. This fraction contained 6·7 % of N and only a trace of P. On hydrolysis with 2·5 % HCl the polypeptide-like fraction of Soru & Combiesco liberates a further ether-soluble constituent and virtually the whole of the N of the substance is liberated as amino-N. The latter observation appears to us surprising in view of the presence in the compound of arginine, and probably other amino-acids containing N other than as amino-groups. The corresponding polypeptide of *B. dysenteriae* (Shiga) contains 11·6 % N, tyrosine and arginine are present and again about 50 % of the total N is liberated as amino-N on acid hydrolysis [Morgan & Partridge, 1940].

Two other components of the antigenic complex have also been isolated but not yet investigated in any detail. Mixed lipid fractions amounting to 3–4 % were isolated in several experiments and appear to consist of a mixture of fatty acids together with a phospholipin. Soru & Combiesco [1940] obtained 15·8 % of an ether-soluble fraction from the *Bact. typhosum* O-antigen on hydrolysis with dilute acetic acid. The fraction was shown to consist of a mixture of fatty acids. Even larger amounts of lipid fractions have been isolated by Boivin & Mesrobeanu [1938, 2] on hydrolysis, with strong HCl, of the isolated O- and Vi-antigens of *Bact. typhosum*, which liberated 27·2 and 26·5 % of 'acides gras', respectively.

An alcohol-soluble nitrogenous fraction which comprises 10–20 % of the antigen has also been obtained. It contains 13·4 % N, the bulk of which is not present as free amino-groups and the percentage of these does not increase markedly on treatment with trypsin or on hydrolysis with acids. The nature of this component is still undetermined, and this problem is under investigation. The parts played by the alcohol-soluble component and the lipids in the complex antigen cannot yet be postulated but it is thought that they are of less fundamental importance than the polysaccharide and polypeptide components. Morgan & Partridge [1940] have shown that, although the *B. dysenteriae* (Shiga) antigen exists in the bacterial cell as a phospholipin-polysaccharide-polypeptide complex, the phospholipin component may be removed leaving a polysaccharide-polypeptide complex which is still specifically antigenic.

Preliminary work on an O-antigenic strain of *Bact. typhi-murium* has yielded similar results to those obtained with *Bact. typhosum* Ty 2, and led to the isolation of a polysaccharide fraction, an insoluble polypeptide-like component, a small amount of a lipid substance and a soluble nitrogenous component.

Summary

1. The *Bact. typhosum* Ty 2 antigen has been shown to consist of the following constituents, into which it may be dissociated on gentle hydrolysis: a polysaccharide component (50–60 %), an insoluble polypeptide (about 16 %), a soluble nitrogenous component (10–20 %) and a small lipid component (3–4 %).

2. The polysaccharide represents the O-specific hapten of the antigen; it has a reducing value on hydrolysis of 83 % in terms of glucose and is highly dextrorotatory ($[\alpha]_D^{20°} = +114°$). It appears to be free from ketose, pentose and uronic acid residues but yields *d*-galactose, *d*-mannose and *d*-glucose on hydrolysis. An identical polysaccharide has been prepared directly from the dried organisms by extraction with dilute acetic acid.

3. The polypeptide component is insoluble in water and soluble in dilute alkalis. It contains 8·2 % N, partly in the form of tyrosine and arginine units and about 50 % of the total N is liberated as amino-groups on acid hydrolysis.
4. The nature of the soluble nitrogenous component is, as yet, undetermined.
5. The antigen of *Bact. typhosum* Ty2 is resistant to tryptic digestion.

These studies and others in the same general field are of necessity extremely expensive. They have involved the growth of relatively enormous masses of pathogenic bacteria and laborious and large-scale chemical manipulations. They have been rendered possible by grants received from many different sources: from the Leverhulme Trust, the Pilgrim Trust, Imperial Chemical Industries, Ltd., and from a fund raised by the late Sir Austen Chamberlain, to which many generous private donors have contributed. One of us (G.G.F.) has held a Grocers' Company Scholarship for Medical Research. To all these we would express our thanks.

The authors wish to thank Prof. H. Raistrick for much valuable advice and criticism.

REFERENCES

Boivin & Mesrobeanu (1934). *C.R. Soc. Biol., Paris*, **115**, 304, 309.
—— —— (1937). *C.R. Soc. Biol., Paris*, **124**, 442.
—— —— (1938, 1). *Ann. Inst. Pasteur*, **61**, 426.
—— —— (1938, 2). *C.R. Soc. Biol., Paris*, **128**, 5.
—— —— & Mesrobeanu (1933). *C.R. Soc. Biol., Paris*, **114**, 307.
—— —— —— (1935). *Arch. roum. Path. exp. Microbiol.* **8**, 45.
Fiske & Subbarow (1925). *J. biol. Chem.* **66**, 375.
Freeman, Challinor & Wilson (1940). *Biochem. J.* **34**, 307.
Jolles & Morgan (1940). *Biochem. J.* **34**, 1183.
Malek (1939). *C.R. Soc. Biol., Paris*, **132**, 15.
Meyer (1938). *C.R. Soc. Biol., Paris*, **129**, 485.
Miles & Pirie (1939, 1). *Brit. J. exp. Path.* **20**, 83.
—— —— (1939, 2). *Brit. J. exp. Path.* **20**, 109.
—— —— (1939, 3). *Brit. J. exp. Path.* **20**, 278.
Mitchell & Hamilton (1929). The Biochemistry of the Amino Acids. New York.
Morgan (1937). *Biochem. J.* **31**, 2003.
—— (1938). *Helv. chim. Acta*, **21**, 469.
—— & Partridge (1940). *Biochem. J.* **34**, 169.
Raistrick & Topley (1934). *Brit. J. exp. Path.* **15**, 113.
Stewart & Hendry (1935). *Biochem. J.* **29**, 1683.
Soru & Combiesco (1940). *C.R. Soc. Biol., Paris*, **133**, 498.
Topley, Raistrick, Wilson, Stacey, Challinor & Clark (1937). *Lancet*, **1**, 252.
White (1929). *J. Path. Bact.* **32**, 85.

60. THE 3:5-DINITROBENZOYL DERIVATIVES OF THE AMINO-ACIDS AND THEIR USE IN SEPARATING THE ISOMERS OF LEUCINE AND VALINE

By BERNARD WILLIAM TOWN

From the Department of Chemistry, The Medical College of St Bartholomew's Hospital, London

(*Received 26 March 1941*)

In recent papers, Saunders [1934; 1938] has described the preparation of the 3:5-dinitrobenzoyl derivatives of certain amino-acids and has suggested their use for purposes of identification. This possibility has been confirmed by the present author; however, certain discrepancies have been observed between published data and present experience and these observations are embodied in this communication.

In the first place, the reaction between 3:5-dinitrobenzoyl chloride and amino-acids has been found to be much slower than stated by Saunders. Reaction times of the order of 30 min. have been found, compared with quoted times of 2 min. for similar quantities of material. It was also observed that some 3:5-dinitrobenzoic acid is always obtained during benzoylation, even when molecular proportions of reactants are employed, and this can be very troublesome. It is suggested by Saunders that practically no 3:5-dinitrobenzoic acid is formed during the reaction.

It has also been found that the melting points of some of the derivatives differ considerably from those quoted by Saunders, and such differences are noted in the experimental section of the paper. Also in the cases of valine and leucine more than one derivative has been isolated. The melting points of the derivatives which the author has been able to prepare are quoted in Table 1. These melting points have been consistently obtained in this laboratory and are believed to be correct.

Table 1. *3:5-Dinitrobenzoyl derivatives of amino-acids*

Derivative of:	M.P. °C
Glycine	182·2, shrinks 181·4
l(+)-Alanine	177, softens 176·6
l(−)-Threonine	181, softens 180
l(−)-Serine	183, shrinks 112
l(−)-Cystine	Derivative not suitable
dl-Valine	211·4, softens 210·8
l(+)-Valine	207
*nor*Valine (?)	182
l(−)-Leucine	188, softens 187
l(+)-*iso*Leucine	170·4, softens 169·2
*nor*Leucine (?)	163
dl-Phenylalanine	161
l(−)-Tyrosine	No derivative obtainable
l(−)-Aspartic acid	184·8, softens 184·4
Na-salt of aspartic acid derivative	218
l(+) / *d*(−) / *dl*-Glutamic acid	104
r-Glutamic acid	204
Synthetic β-hydroxyglutamic acid	229·5, darkens 228
3:5-Dinitrobenzoic acid	207·8
3:5-Dinitrobenzoyl chloride	74

A derivative has been isolated from aspartic acid, whereas Saunders isolated no derivative, but deduced from a formaldehyde titration that 50 % of the aspartic acid remained unchanged. Experience has shown that this derivative is very easily hydrolysed and may be decomposed if it is kept in a solution more acid than *p*H 2 for any length of time.

The crystalline forms of these derivatives are described in the experimental section, and photographs of most of them have been taken to ensure a more reliable record.

As many of the derivatives have similar melting points, determination of melting point alone is not sufficient to characterize them. Their melting points are, however, very sensitive to the presence of impurities, particularly 3:5-dinitrobenzoic acid, and it is convenient to prepare standard preparations of all these derivatives so that mixed melting points can be taken.

A very important observation has been made in that the various isomeric valines and leucines can be separated by the fractional precipitation of their 3:5-dinitrobenzoyl derivatives at different *p*H. No such separation could be effected by fractional crystallization. The great value of this separation lies in the fact that no means of estimating the proportion of the different isomers of valine and leucine in a mixture has hitherto been available. It was during the course of such separation that derivatives were isolated from valine and leucine which differed from the normal in melting point, but were yet pure. It is suggested that they are the derivatives of *nor*valine and *nor*leucine. This is subject to confirmation. They are indicated by a query in Table 1.

It has been found very difficult to free any derivative from traces of 3:5-dinitrobenzoic acid once it has been contaminated. In every case, therefore, precipitation of the 3:5-dinitrobenzoyl derivative has been effected gradually by adding standard acid to the alkaline solution in stages, filtering off each precipitate as it forms, and recording the *p*H by means of indicators. The derivatives of dicarboxylic amino-acids are insoluble in benzene; this enables any free 3:5-dinitrobenzoic acid to be separated by continuous extraction in a Jena sintered glass apparatus as used for rubber analysis. This treatment was not suitable for the derivatives of monobasic monoamino-acids.

Experimental

3:5-Dinitrobenzoyl chloride hydrolyses very rapidly on exposure to air. It is best to obtain a freshly crystallized specimen and put it up in 1 g. portions in dry tubes immediately; such a specimen has been kept in good condition for more than a year. When pure it crystallizes in needles and melts at 74°.

3:5-Dinitrobenzoic acid which is always obtained as a by-product crystallizes in thick rectangular plates, M.P. 207·8°.

In all cases described below 1 g. of 3:5-dinitrobenzoyl chloride was used, and was added slowly, with mechanical shaking between each addition, to the calculated amount of amino-acid in 5 ml. water, containing the calculated quantity of 2*N* NaOH (2 mol.). Excess of acid chloride was not used owing to the difficulty of separating free 3:5-dinitrobenzoic acid from the amino-acid derivative.

Derivatives of individual amino-acids

Glycine. On acidifying the reaction mixture with *N* HCl to *p*H 3·3 a fine crystalline precipitate was obtained, M.P. 158–160°. Crude yield 1·06 g. (91 % of theory). It was found difficult to separate from traces of 3:5-acid by crystallization, but after crystallizing twice from 10 % acetic acid and once from water, it gave long fine needles, M.P. 182·2°, shrinking at 181·4°.

Loss in wt. on drying at 110° = 6·5 % (theory for $C_9H_7O_7N_3$, H_2O, 6·27 %). Saunders [1934] quotes M.P. 179°.

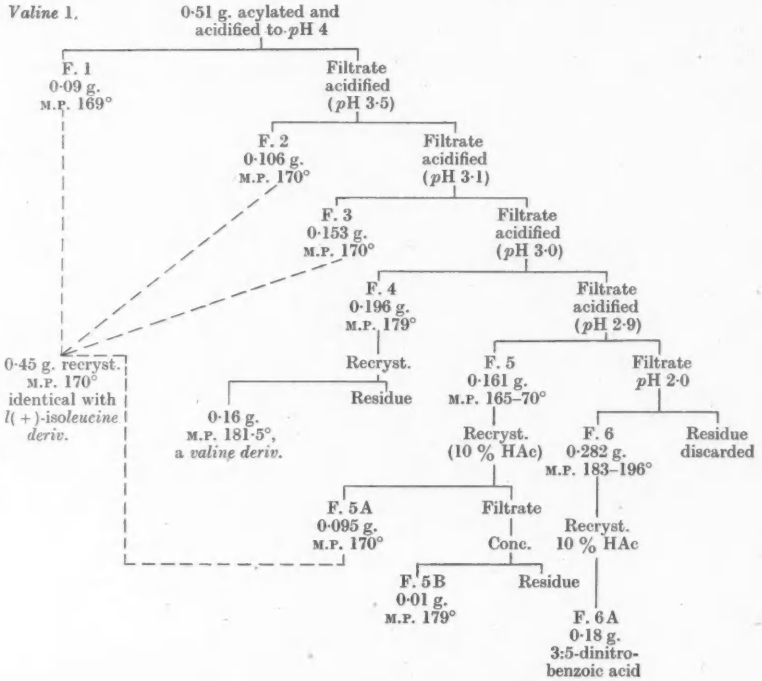
l(+)-*and* dl-*alanine*. On acidifying the solution after benzoylation of *dl*-alanine (B.D.H.) to *p*H 2·6, and leaving in the refrigerator overnight, 0·729 g. of derivative, M.P. 176°, softening at 172° was obtained (63 % yield). After crystallization from 10 ml. 10 % acetic acid it yielded 0·61 g., M.P. 177° sharp, containing no water of crystallization.

A sample of *l*(+)-alanine also gave a derivative with the same melting point (58 % yield); mixed M.P. with the *dl*-derivative, 174°, softening at 172°. The above data agree with those quoted in the literature.

Valine. Great discrepancies have been observed here between the figures quoted in the literature and those actually obtained. The derivative has been prepared from three samples of valine, first from a specimen of *dl*-valine, and then from two samples of *l*(+)-valine which had been presented to the author.

dl-*Valine* (B.D.H.) gave, on bringing the reaction mixture to *p*H 2·8, a crude product M.P. 184° (68·5 % yield). This, after purification, crystallized in glistening flat rectangular platelets, M.P. 211·4°, softening at 210·8°. (Found: C, 46·45; H, 4·27; N, 13·42 %. $C_{12}H_{13}O_7N_3$ requires C, 46·35; H, 4·18; N, 13·50 %.) This derivative contains no water of crystallization, whereas Saunders found for the derivative of *l*(+)-valine M.P. 157–158°, and also 1 mol. of water of crystallization.

l(+)-*Valine*. As both samples of natural valine proved to be mixtures and as the results differed with each sample, it will be easier to record the findings diagrammatically.


Valine 1.
0·51 g. acylated and acidified to *p*H 4
F. 1 0·09 g. M.P. 169°
Filtrate acidified (*p*H 3·5)
F. 2 0·106 g. M.P. 170°
Filtrate acidified (*p*H 3·1)
F. 3 0·153 g. M.P. 170°
Filtrate acidified (*p*H 3·0)
F. 4 0·196 g. M.P. 179°
Filtrate acidified (*p*H 2·9)
0·45 g. recryst. M.P. 170° identical with *l*(+)-isoleucine *deriv.*
Recryst.
Residue
F. 5 0·161 g. M.P. 165–70°
Filtrate *p*H 2·0
0·16 g. M.P. 181·5°, a *valine deriv.*
Recryst. (10 % HAc)
F. 6 0·282 g. M.P. 183–196°
Residue discarded
F. 5A 0·095 g. M.P. 170°
Filtrate
Conc.
Recryst. 10 % HAc
F. 5B 0·01 g. M.P. 179°
Residue
F. 6A 0·18 g. 3:5-dinitrobenzoic acid

In all, taking into account various minor fractions, there were isolated: 0·508 g. *l*(+)-*iso*leucine derivative, M.P. 170·2°, equivalent to 0·205 g. *l*(+)-*iso*leucine or 40 % of the initial material; 0·196 g. valine derivative, M.P. 181·5°, equivalent to 0·074 g. of valine or 14·5 % of the starting material.

As the total yield of derivatives (54·5 %) is of the same order as the yield normally obtained in an acylation with 3:5-dinitrobenzoyl chloride, it seems reasonable to suppose that this sample of so-called valine was in reality a mixture of *iso*leucine and valine in the proportion of approximately 74 % *iso*leucine and 26 % valine.

It is important to note that the M.P. of this valine derivative is far removed from that obtained with *dl*-valine (B.D.H.) and also from that obtained by Saunders [1934] who gives M.P. 157–158°.

Analysis of these two derivatives by Schoeller gave the following results: *iso*leucine derivative: found: C, 47·87; H, 4·40; N, 13·04 %; calc. C, 48·00; H, 4·62; N, 12·92 %; valine derivative: found: C, 46·57; H, 4·36 %, N, 13·35 %; calc. C, 46·35; H, 4·36; N, 13·50 %.

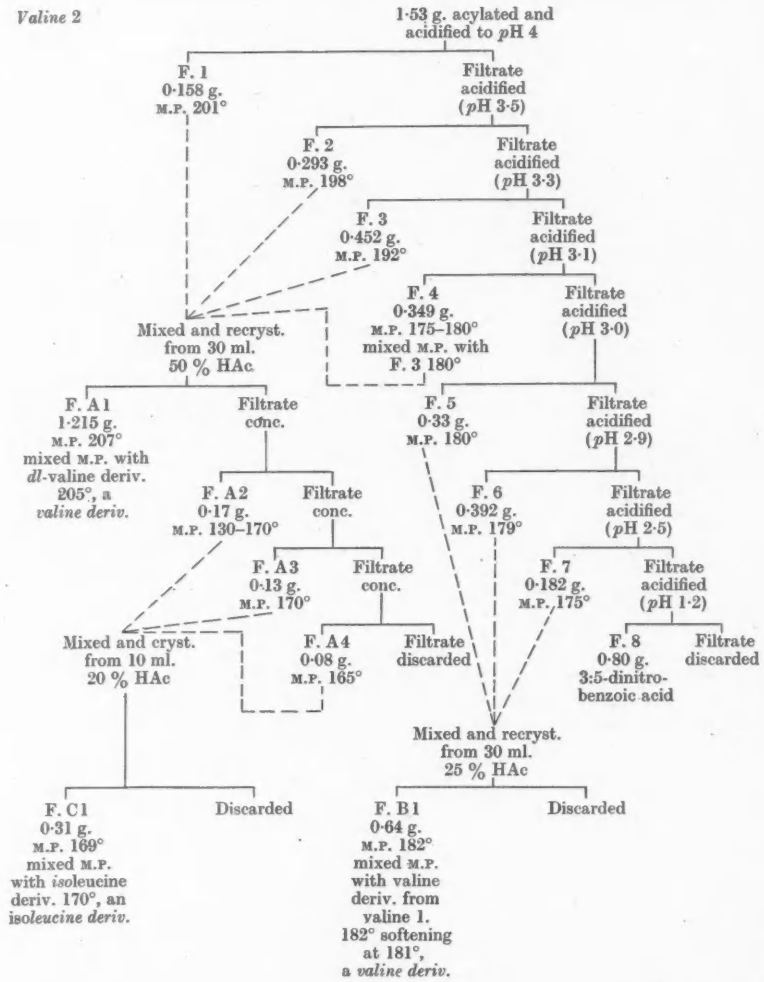
Valine 2. From this sample there have been isolated (see diagram): 0·31 g. of *iso*leucine derivative, M.P. 170°, equivalent to 0·115 g. of free *iso*leucine or 7·5 % of the initial weight of substance; 0·64 g. of a valine derivative, M.P. 182°, equivalent to 0·26 g. of free valine, or 17 % of the starting material (this derivative is identical with that isolated from valine 1); 1·22 g. of another valine derivative, M.P. 207°, equivalent to 0·458 g. of free valine or 30 % of the starting material; this valine derivative is apparently identical with that obtained from synthetic *dl*-valine (B.D.H.) as no lowering of M.P. occurs on mixing. As this synthetic valine is certainly that variety with a *gem*-dimethyl group, it is possible to fix the structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 207°. The structure of the valine giving the 3:5-dinitrobenzoyl derivative, M.P. 182°, remains to be determined. It is suggested provisionally that this is *nor*valine.

The three derivatives isolated from valine 2 were analysed by Schoeller: 3:5-dinitrobenzoyl derivative, M.P. 182°: C, 46·52; H, 4·39; N, 13·31 %; 3:5-dinitrobenzoyl derivative, M.P. 207°: C, 46·48; H, 4·23; N, 13·50; valine derivative requires C, 46·35; H, 4·18; N, 13·50 %; 3:5-dinitrobenzoyl derivative, M.P. 170°: C, 47·78; H, 4·42; N, 13·02 %; *iso*leucine derivative requires C, 48·00; H, 4·62; N, 12·92 %. The 3:5-dinitrobenzoyl derivative, M.P. 182°, crystallizes in very long but narrow rectangular rods, while the derivative with M.P. 207° crystallizes in large thin hexagonal plates which often have an irregular outline The derivative from *dl*-valine, M.P. 211·4°, crystallizes in the same form as the derivative with M.P. 207°, which confirms their identity. Photographs have been taken of these crystals.

It is interesting to compare the yields of 3:5-dinitrobenzoyl derivatives from these two samples of valine:

	3:5-Dinitrobenzoyl derivatives isolated			
	M.P. 207° %	M.P. 182° %	M.P. 170° %	Total %
Valine 1	0	14·5	40	54·5
Valine 2	30	17	7·5	54·5

The yield in either case is 54·5 % expressed as amino-acid isolated in terms of starting material. This yield is of the same order as is usually obtained in acylating pure amino-acids, so that if no other amino-acids are assumed to be


Valine 2
1·53 g. acylated and acidified to pH 4
F. 1 0·158 g. M.P. 201°
Filtrate acidified (pH 3·5)
F. 2 0·293 g. M.P. 198°
Filtrate acidified (pH 3·3)
F. 3 0·452 g. M.P. 192°
Filtrate acidified (pH 3·1)
F. 4 0·349 g. M.P. 175–180° mixed M.P. with F. 3 180°
Filtrate acidified (pH 3·0)
Mixed and recryst. from 30 ml. 50 % HAc
F. A1 1·215 g. M.P. 207° mixed M.P. with dl-valine deriv. 205°, a valine deriv.
Filtrate conc.
F. 5 0·33 g. M.P. 180°
Filtrate acidified (pH 2·9)
F. A2 0·17 g. M.P. 130–170°
Filtrate conc.
F. 6 0·392 g. M.P. 179°
Filtrate acidified (pH 2·5)
F. A3 0·13 g. M.P. 170°
Filtrate conc.
F. 7 0·182 g. M.P. 175°
Filtrate acidified (pH 1·2)
Mixed and cryst. from 10 ml. 20 % HAc
F. A4 0·08 g. M.P. 165°
Filtrate discarded
F. 8 0·80 g. 3:5-dinitro-benzoic acid
Filtrate discarded
Mixed and recryst. from 30 ml. 25 % HAc
F. C1 0·31 g. M.P. 169° mixed M.P. with isoleucine deriv. 170°, an isoleucine deriv.
Discarded
F. B1 0·64 g. M.P. 182° mixed M.P. with valine deriv. from valine 1. 182° softening at 181°, a valine deriv.
Discarded

present, it is possible to calculate the original percentage composition of the mixtures:

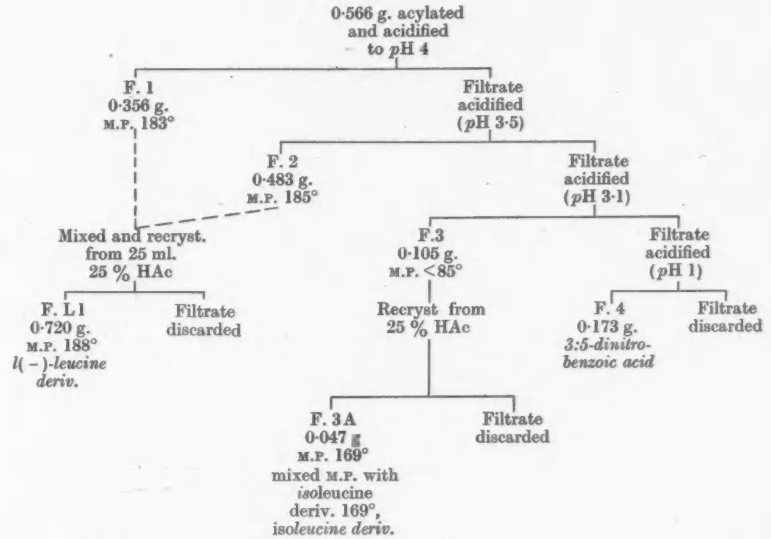
	Valine 1 %	Valine 2 %
Valine	Nil	55
*nor*Valine (?)	26·6	31·2
*iso*Leucine	73·4	13·8
	100·0	100·0

While these figures are unlikely to be accurate to more than 1–2 %, they do give some idea of the compositions of the two samples and indicate that neither

was pure. Until methods for the isolation of amino-acids have been perfected it would seem idle to give tables showing the percentage composition of proteins.

The melting point quoted by Saunders for the valine derivative is 157–158°, and he states that it crystallizes with 1 H_2O. The two derivatives isolated in the present work are anhydrous, and neither melting point is in the vicinity of that quoted.

l(−)-*Leucine*. For this preparation a sample of *l*(−)-leucine was obtained from Schuchardt's, and was of guaranteed purity.


0·566 g. acylated and acidified to pH 4
F. 1 0·356 g. M.P. 183°
Filtrate acidified (pH 3·5)
F. 2 0·483 g. M.P. 185°
Filtrate acidified (pH 3·1)
Mixed and recryst. from 25 ml. 25 % HAc
F. 3 0·105 g. M.P. <85°
Filtrate acidified (pH 1)
F. L 1 0·720 g. M.P. 188° l(−)-leucine deriv.
Filtrate discarded
Recryst from 25 % HAc
F. 4 0·173 g. 3:5-dinitrobenzoic acid
Filtrate discarded
F. 3 A 0·047 g. M.P. 169° mixed M.P. with isoleucine deriv. 169°, isoleucine deriv.
Filtrate discarded

This *l*(−)-leucine derivative had M.P. 188°, softening at 187·2°, on a standard thermometer; it contained no water of crystallization. These data agree with those quoted by Saunders.

The yield obtained was 61 % of theory; there was also obtained 3·4 % of *l*(+)-*iso*leucine derivative, M.P. 169°. Thus this amino-acid, in spite of its label, was not pure and consisted of a mixture of 95 % *l*(−)-leucine and 5 % *l*(+)-*iso*leucine. A sample of leucine isolated from the hydrolysis of horsehair was also used to prepare the derivative as this was a specimen thought to be of great purity.

From 1·132 g. of this there were isolated 1·32 g. leucine derivative, M.P. 188°, equivalent to 0·532 g. leucine or 47·2 % of the original material. This derivative crystallizes in very thin rectangular plates with one or both ends cut off obliquely.

There was also isolated another derivative, M.P. 163°, also crystallizing in very thin rectangular plates, but with all ends perfectly square, in amount corresponding to 0·097 g. leucine or 8·55 % of the starting material. (Found: C, 47·75; H, 4·60; N, 13·02 %. Theory: C, 48·00; H, 4·61; N, 12·91 %.)

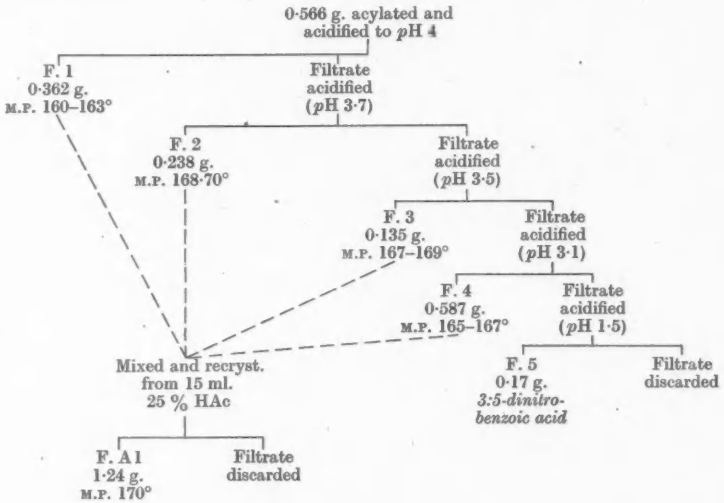
The total yield of leucine derivatives from this specimen of leucine is thus 55·7 % of the initial leucine which is of the same order as is usually obtained.

One may calculate on this basis that the composition of this sample of leucine was approximately:

	%
l(−)-Leucine	84·8
*nor*Leucine (?)	15·2
	100·0

It is assumed provisionally that this derivative, which analyses for a leucine derivative but does not correspond with that either of leucine or *iso*leucine, is that of *nor*leucine.

l(+)-iso*Leucine*. A sample of pure *l*(+)-*iso*leucine from Schuchardt was used for the preparation of this derivative.



This derivative crystallizes in long narrow rectangular plates. A derivative was also prepared from the *iso*leucine isolated from gliadin by Town [1929], and this also had M.P. 170°.

Serine. A sample of pure serine isolated by the author from gliadin was used in this preparation. 0·457 g. serine was acylated, but as the derivative did not precipitate until the solution was acidified to *p*H 1, it was contaminated with free 3:5-dinitrobenzoic acid which was difficult to remove by crystallization from 25 % acetic acid (10 ml.). The weight of crude derivative was 0·964 g. and its M.P. 157°, softening at 140°. It was extracted continuously with benzene in a Jena sintered glass extraction thimble for 45 min. The benzene on evaporation yielded 0·32 g. 3:5-dinitrobenzoic acid. The residue insoluble in benzene, 0·61 g., M.P. 183°, was crystallized from water and then had M.P. 183°, softening at 181° and shrinking at 112°, crystallizing in needles. Yield 46·8 %. This serine derivative was dried at 110°: loss in weight, 5·45 %; calc. for $C_{10}H_9N_3O_8$, H_2O, 5·66 %. (Found: C, 37·99; H, 3·77; N, 13·22 %; calc. for hydrated serine derivative: C, 37·90; H, 3·37; N, 13·24 %. The anhydrous derivative has M.P. 183°. Saunders quotes the melting point of serine derivative as 94–95°.

l(−)-*Threonine.* A pure sample of this amino-acid isolated by the author from gliadin was used for the preparation of the derivative. The same difficulty was encountered as in the preparation of the serine derivative, that the solution had to be acidified to *p*H 2 before any precipitate was formed. On leaving in the ice-chest for some hours a precipitate of 0·07 g. 3:5-dinitrobenzoic acid formed. The solution had now to be left for over a week.

117 mg. of threonine were benzoylated, and 79 mg. of a derivative, M.P. 135°, slowly separated. It was extracted continuously with benzene and gave 34 mg. residue, which after crystallizing from 5 ml. 10 % acetic acid had M.P. 181°, softening at 180°. It crystallized in long thin rectangular rods. Yield 11·2 %. (Found: C, 41·83; H, 3·60; N, 13·30 %; calc. for $C_{11}H_{11}N_3O_8$: C, 42·18; H, 3·51; N, 13·42 %.)

The yields of derivatives of both these hydroxy-acids are low, presumably owing to their greater solubilities.

Cystine. The derivative is not really suitable for the identification of cystine, and although the author's experience differs rather from the description of Saunders, its description is unwarranted.

Phenylalanine. dl-Phenylalanine (B.D.H.), 0·715 g., was acylated, and after bringing to *p*H 7, an oil was precipitated. Sufficient methyl alcohol was added just to dissolve the oil and the solution was left for 24 hr.; 0·90 g. crystals separated, M.P. 147°, softening at 142°. The filtrate was brought to *p*H 4 and treated in the same way; a second crop was obtained, 0·22 g., M.P. 110°, softening at 105°. Only 3:5-dinitrobenzoic acid was obtained on further acidification.

The mixed crops were crystallized from 30 ml. 60 % methyl alcohol and gave 0·78 g., M.P. 160°, softening at 107°. Yield 50·3 %. On recrystallization from 25 ml. 50 % acetic acid, the derivative had M.P. 161°, softening at 160°, and crystallized in thin rectangular rods; wt. 0·57 g. The substance has no water of crystallization. (Found: C, 53·64; H, 3·90; N, 11·60 %; calc. for $C_{16}H_{13}N_3O_7$: C, 53·50; H, 3·62; N, 11·70 %.)

Saunders quotes M.P. 93°, and states that the compound crystallizes with 0·5 mol. H_2O.

Glutamic acid. Natural glutamic acid hydrochloride (0·797 g.) isolated by the author from gliadin was used and, after acylation, the solution was acidified to *p*H 2. A slight oily precipitate separated which rapidly solidified and, after 12 hr. in the ice-chest, 1·073 g. of derivative were obtained, M.P. 130°, softening at 102°. The derivative is quite insoluble in benzene, and after extracting continuously for 1 hr. with boiling benzene the residue (0·570 g.) had M.P. 155°, softening at 153°. The benzene on evaporation gave 0·448 g. of 3:5-dinitrobenzoic acid.

The residue insoluble in benzene was crystallized from 8 ml. 10 % acetic acid and yielded 0·46 g. of a derivative, M.P. 104°, softening at 102°, crystallizing in very long fine needles. It has a water content of 5·47, 5·45 %; $C_{12}H_{11}N_3O_9$, H_2O requires 5·04 %. The anhydrous derivative has M.P. 182°, softening at 179°. The yield of derivative is 38·4 % of theory. Saunders quotes M.P. 98–99° and finds that it crystallizes with 0·5 ml. H_2O.

From the mother liquors from the separation of glutamic acid as its hydrochloride from a protein hydrolysate, the author has isolated a glutamic acid which yields a 3:5-dinitrobenzoyl derivative, M.P. 204°, and crystallizes in large but very thin hexagonal plates which tend to overlay one another. It crystallizes in the anhydrous condition. This is believed to be the derivative of *r*-glutamic acid; it is certainly not that of *dl*-glutamic acid which gives the derivative M.P. 104° [Town, 1941].

Aspartic acid. Natural aspartic acid (0·58 g.) was acylated and acidified to *p*H 2, when 0·27 g. of 3:5-dinitrobenzoic acid separated. The filtrate was left for 12 hr. in the ice-chest and 0·36 g. of derivative obtained, M.P. 180°, softening at 177°. This was crystallized from 3 ml. 10 % acetic acid in which it was easily soluble: 0·13 g., M.P. 184·8°, softening at 184·4°, shrinking slightly at 100°. From the mother liquor there was also isolated 0·017 g. of the glutamic acid derivative, M.P. 204°, identical with that isolated from the dicarboxylate fraction of gliadin [Town, 1941]. This has been obtained in small quantity from all the specimens of natural aspartic acid (three) which the author has examined.

The aspartic acid derivative readily crystallizes from water, in which, however, it is rather soluble; it is readily decomposed in solutions more acid than *p*H 2. The substance crystallizes with 1 H_2O: loss in weight at 110°, 5·65 %, calc. for $C_{11}H_9N_3O_9$, H_2O 5·22 %. The anhydrous material has C, 40·1; H, 2·90; N, 12·87 %; calc. for $C_{11}H_9N_3O_9$, C, 40·40; H, 2·75; N, 12·84 %. The yield of derivative is only 25 %, this low figure probably being due to its solubility and to its ready decomposition in acid solution.

In a repetition of this experiment, the solution after benzoylation was only acidified to *p*H 4, when a glistening white precipitate formed consisting of a mass of fine rectangular plates, 0·692 g., M.P. 220°. This was very soluble in water, and originally must have been salted out by the other sodium salts present. It dissolved in 4 ml. H_2O on warming, and separated in shining flat plates on cooling, wt. 0·32 g. (Found: C, corrected for C in ash 37·4; H, 3·09; N, 11·71; Na, 6·66 %; the mono Na salt of 3:5-dinitrobenzoylaspartic acid, $C_{11}H_8N_3O_9Na$, requires C, 37·80; H, 2·30; N, 12·02; Na, 6·60 %.) No other 3:5-dinitrobenzoyl derivative has been found to give a sodium salt which can be salted out in this manner.

β-Hydroxyglutamic acid. A sample of synthetic β-hydroxyglutamic acid (0·232 g.) kindly presented by Prof. Harington was used in this experiment.

After benzoylation, the solution was acidified to *p*H 1 and left for 12 hr. in the ice-chest. The derivative was filtered, wt. 0·426 g., and extracted continuously with benzene. The benzene extract gave 0·30 g. 3:5-dinitrobenzoic acid, and the residue, 0·120 g., M.P. 220–221°, was dissolved in 10 ml. absolute methyl alcohol, filtered from a trace of ferric iron impurity, freed from methyl alcohol and crystallized from 12 ml. 25 % acetic acid. It crystallized in long fine needles, wt. 0·116 g., M.P. darkening at 228° and melting with decomposition at 229·5°. Yield 27 % of theory. (Found: C, 40·39; H, 3·12; N, 11·70 %; calc. for $C_{12}H_{11}N_3O_{10}$, C, 40·40; H, 3·08; N, 11·78 %.)

Summary

It has been confirmed that 3:5-dinitrobenzoyl chloride is a very valuable reagent for the identification of amino-acids. A table of melting points of those derivatives which the author has been able to prepare is given.

The melting points found for some derivatives, notably those of phenylalanine, serine and valine, differ considerably from those quoted by Saunders.

There have been isolated two derivatives from the specimens of valine available, and three derivatives from the samples of leucine at the author's disposal. These are thought to be the derivatives of valine and *nor*valine, and of leucine, *iso*leucine and *nor*leucine respectively. The identity of the *nor*valine and *nor*leucine derivatives is subject to confirmation.

It has been found possible to separate the components in mixtures of the various isomers of valine and leucine by fractional precipitation of their deri-

vatives at differing *p*H. This is important, as no means of separating mixtures of such isomers has hitherto been available.

A derivative has been isolated from aspartic acid, of which the Na salt is readily salted out at *p*H 4. The aspartic acid derivative is easily hydrolysed in solutions more acid than *p*H 2.

No derivative could be isolated from tyrosine.

REFERENCES

Saunders (1934). *Biochem. J.* **28**, 580.
—— (1938). *J. chem. Soc.* p. 1397.
Town (1929). *J. Soc. chem. Ind., Lond.*, **48**, 1248.
—— (1941). *Biochem. J.* **35**, 417.

61. ON THE METHOD OF FORMATION OF PYRUVIC ACID BY BARLEY

By WILLIAM OWEN JAMES, GLADYS MACPHAIL JAMES and ARTHUR HUGH BUNTING[1]

From the Department of Botany, Oxford

(*Received 20 March 1941*)

Pyruvic acid is consumed in barley respiration [James & Norval, 1938] and can also be made to accumulate by poisoning the living plant with acetaldehyde or aromatic sulphonic acids [James & James, 1940]. Organic phosphates are present [Arney, 1939] which appear to be connected with respiration in a phosphorylating cycle [James & Arney, 1939]. It is, therefore, a matter of interest to discover whether the sugars consumed in barley respiration pass through such a cycle in their conversion into pyruvic acid.

Material

Leaves of young barley plants (var. Plumage Archer) were cut and frozen overnight at about $-12°$. They were allowed to thaw at room temperature and their sap was squeezed out through muslin by hand. This sap was usually allowed to stand in the refrigerator (at about 2°) for 24 hr. before use. Any sediment which formed was filtered off, a clear yellow liquid resulting. In a few experiments, as will be described, further purification was attempted. None of these saps by themselves yielded appreciable amounts of pyruvic acid, even after incubation (see p. 589) under the standardized experimental conditions, and they showed very little carboxylase activity. They were, therefore, very convenient for investigating the formation of pyruvic acid from the substances which it was desired to examine. Intact tissues could not be used since they would not have taken up the substrates sufficiently quickly.

Methods

Extraction. After a period of incubation at 30° in the presence of thymol as antiseptic, usually for 48 hr., any pyruvic acid present was separated from the sap. 10 ml. barium acetate were pipetted into the digest and the reaction adjusted to *p*H 8 by running in *M*/5 baryta. The barium precipitate was centrifuged off and another drop of barium acetate added, to make sure that precipitation was complete. 25 % trichloroacetic acid was then added, to give a final concentration of 5 %. The mixture was allowed to stand for 15 min. and then brought to the boil. The protein flocculum was filtered off and the clear filtrate transferred to a large distillation flask. Distillation was carried out under reduced pressure with a fine stream of air passing through the solution. Water and other volatile substances came over at 50–55° and about 50 mm. pressure.

Identification. Pyruvic acid was identified in the distillate by the isolation of its 2:4-dinitrophenylhydrazone by the method of Peters & Thomson [1934]. The hydrazones obtained were compared with an authentic sample from twice redistilled pyruvic acid, giving M.P. 216°, a canary yellow colour and diamond-

[1] Rhodes Scholar, 1938–41.

shaped platelets, single and clustered. This hydrazone gave a deep red coloration with alcoholic KOH, which was not discharged by adding water.

The separation of the pure substance from barley preparations presents numerous difficulties, and it was commonly found that the final product showed orange-tinted zones on crystallizing out. To avoid further laborious purifications, well-formed crystals were selected mechanically from the pure yellow zones for determination of the melting-point. Even when the quantity available was fairly considerable, it was found convenient to use the hot-box already described [James & James, 1940], selecting a small number of characteristic crystals for the purpose. These were either allowed to crystallize out on a coverslip placed in the crystallizing dish, or were scraped up and placed upon it with a clean scalpel.

Enzyme block. Loss of pyruvic acid might be expected, principally from the activity of carboxylase [James & Norval, 1938]. Barley saps as used in the present experiments released CO_2 from added pyruvic acid. The release was slow and was shown, in another series of experiments, to be much less than when solid leaf material was present.

Low concentrations of 1-naphthol-2-sulphonic acid were used to restrict this carboxylase activity. Complete inhibition was not attempted, since the necessary concentration of the naphtholsulphonic acid caused heavy precipitations in the sap. High percentage yields of pyruvic acid could not, therefore, be expected, and were not obtained. Losses due to dephosphorylation of substrates by phosphatases are also known to occur [James & Heard, 1940].

Experiments

Sap only. (*a*) *Exp. 48.* A sample sap prepared as above was precipitated with *N*/5 barium acetate (1 part acetate to 20 parts sap). The precipitate was centrifuged off and the excess barium removed with a few drops of saturated ammonium sulphate. 5 ml. clear filtrate were added to 1 ml. 0·2 % 1-naphthol-2-sulphonic acid, and made up to 10 ml. with glass-distilled water. Crushed thymol (3 parts per 1000) was shaken with the digest which was put in a conical flask plugged with cotton-wool and incubated at 30° for 48 hr. No alkali-soluble 2:4-dinitrophenylhydrazone could be isolated.

(*b*) *Exp. 70A.* A sample of sap from young and vigorously growing seedlings was prepared in the usual way and was used immediately, without either barium precipitation or standing at low temperature for 24 hr. 40 ml. were mixed with 5 ml. 1 % sulphonic acid and 5 ml. glass-distilled water. Crushed thymol (3 per 1000) was added and incubation carried out as before. On extraction by the usual method, a small quantity of a 2:4-dinitrophenylhydrazone, soluble in alkali, was obtained. It was red in colour and crystallized poorly, but a narrow zone of spindle-shaped crystals was found which gave a rather indefinite M.P. 205–208°. These results suggest that small amounts of hydrazone-forming substances had been isolated, probably including traces of pyruvic acid. Since it was already known that under suitable conditions barley could be induced to accumulate pyruvic acid, no attempt was made to obtain definite yields in these digests. The absence of pyruvic acid from freshly extracted saps has already been shown [James & Norval, 1938; James & James, 1940].

Sucrose. Exp. 50. Incubation under the usual conditions was carried out with 24 ml. sap, 3 ml. 20 % sucrose (B.D.H. Analar) and 0·3 ml. 0·2 % sulphonic acid. Crushed thymol was added. The sap used in this experiment was precipitated with barium, as described under (*a*) above. This treatment might be

expected to reduce the concentration of phosphate, free and combined, in the sap. No trace of alkali-soluble hydrazones could be isolated.

Glucose and fructose. Special experiments with additions of glucose and fructose were not carried out, since these sugars were known to be present in fresh sap in fair quantity. Their presence did not, in itself, lead to the formation of appreciable quantities of pyruvic acid.

Adenylic acid. Exp. 68B. This experiment was carried out with sap from young green seedlings. The sap was filtered and allowed to stand at 2° for 3 days before use. Its sugar content might be expected to be low. 40 ml. sap were incubated for 48 hr. at 30° with 5 mg. adenylic acid and 0·36 ml. 3 % 1-naphthol-2-sulphonic acid, in the presence of thymol (3 per 1000). It was cleared and distilled and the 2:4-dinitrophenylhydrazones formed in the usual way. The yield was small, with a narrow zone of canary-yellow crystals, probably the pyruvic acid derivative, round the edge. The bulk of the product was orange-coloured and oily. The result was markedly similar to that obtained with fresh sap only (see (*b*) p. 589).

Sucrose + adenylic acid. Exp. 68A. This experiment was carried out simultaneously with the adenylic acid experiment, with an equal aliquot of the same sap. All conditions were identical, except for the addition of 1 g. sucrose to this second digest. A much increased yield of alkali-soluble hydrazone was obtained which crystallized out wholly, though the last crystals to form were somewhat orange in colour. There were numerous bright yellow crystals of the spindle to diamond shape characteristic of the pyruvic acid hydrazone. They gave a deep red colour with alcoholic KOH, which was not discharged on adding water. M.P. 218°, corrected.

Glucose + adenylic acid. Exp. 69. A further supply of sap was prepared exactly as before and used after 24 hr. standing at low temperature. It was possible to use a rather larger digest: 70 ml. sap + 5 mg. adenylic acid + 1·75 g. glucose (B.D.H. analar) + 0·63 ml. 3 % 1-naphthol-2-sulphonic acid + thymol (3 per 1000). The result was similar to that of the previous experiment. A good yield was obtained of characteristic canary-yellow crystals, slightly contaminated with orange, which gave M.P. 216·5°, corrected, and the usual colour reaction with KOH.

Hexosediphosphate. A commercial sample of barium hexosediphosphate containing inorganic phosphate as impurity was used. The ratio of unhydrolysable P to P hydrolysed in 3 hr. was 0·64 as compared with 0·66 calculated from Lohmann's analyses of purified hexosediphosphate. We are indebted to Mr C. R. C. Heard for these figures. Immediately before use, aliquots of the barium salt were dissolved in dilute HCl and the barium centrifuged off after precipitation by a little saturated ammonium sulphate. The solution was neutralized (purple to litmus) with a few drops of strong KOH and made up to volume.

Exp. 47. A preliminary experiment was carried out using 20 ml. of clarified sap (as in (*a*) p. 589) + 4 ml. of a hexosediphosphate solution equivalent to 0·4 g. barium salt + 4 ml. 0·2 % 1-naphthol-2-sulphonic acid + crushed thymol (3 per 1000). The digest was made up to 40 ml. with glass-distilled water. Incubation was carried out under the usual conditions and hydrazones were prepared. There was a small yield of bright yellow crystals, some developing typical diamond forms. They gave a permanent red colour with Lu's [1939] reagent (6 vol. 10 % Na_2CO_3 + 4 vol. *N* NaOH). There was not sufficient for the determination of a melting-point by the usual laboratory method, and the hot-box for micro-determinations had not been prepared when this experiment was

carried out. A melting-point was, therefore, not obtained; but there seems little doubt that a small quantity of pyruvic acid had been isolated. Since the clarified sap used in this experiment gave an entire blank when incubated alone (p. 589), it is reasonable to assume that the pyruvic acid originated from the added hexosediphosphate. Confirmation was afforded by an experiment on a rather larger scale.

Exp. 54. 40 ml. sap + 10 ml. hexosediphosphate solution (≡0·1 g. Ba hexosediphosphate) + 6 ml. 0·2 1-naphthol-2-sulphonic acid + thymol were incubated for 48 hr. at 30°. Isolation of alkali-soluble hydrazones was carried out as usual, yielding a considerable deposit of crystals, mostly canary-yellow, though a little reddish in places. Alcoholic KOH gave a permanent deep red colour which was not discharged on the addition of water. The abundant deposit was largely 'run together' but good diamond-shaped platelets were formed in the sparser zones. These gave M.P. 216°, corrected.

Phosphoglycerate. A pure preparation of the crystalline acid barium salt was given us by Prof. R. A. Peters, to whom we wish to express our gratitude. It was brought into solution in the same way as the hexosediphosphate, immediately before use.

Exp. 67. This experiment was carried out using an aliquot of the same lot of sap as was used for the adenylic acid and adenylic acid + sucrose experiments. 4 ml. phosphoglycerate solution (≡30 mg. BaH_2 phosphoglycerate) and 0·72 ml. 3 % 1-naphthol-2-sulphonic acid were made up to 1000 ml. with sap. After the usual incubation, the preparation and isolation of alkali-soluble hydrazones were carried out, yielding a deposit of yellow crystals. There were crystal clusters and single crystals of typical pyruvate form, giving the usual permanent red colour with KOH; M.P. 216°, corrected.

Lactic acid. Lactic acid is not oxidized by barley saps alone, but on addition of ascorbic acid rapid oxidation takes place [James & Cragg, 1940]. The product of this oxidation was identified as pyruvic acid by means of the above technique, slightly modified.

Exp. 38. 200 ml. sap, clarified as in (*a*), were incubated with 40 ml. *N*/5 lactic acid + 40 ml. *N*/10 ascorbic acid (B.D.H.) + 4 ml. sulphonic acid + 116 ml. phosphate buffer, *p*H 7·0 (= 400 ml. in all). Incubation lasted 17 hr. at 30°. The digest was then killed with 5 % trichloroacetic acid. After it had stood overnight in a refrigerator, 25 % trichloroacetic acid was added to a final concentration of 5 %. After further standing, the digest was brought to the boil and distilled. A fraction coming over at 50–55° and 20 mm. was taken and treated with an amount of 2:4-dinitrophenylhydrazine, which proved to be inadequate. When the hydrazone was finally separated it was oily, and there was a strong smell of free pyruvic acid. Further hydrazine was added and a plentiful precipitate of yellow crystals was finally obtained. They had typical pyruvate forms, gave deep red coloration with alcoholic potash and had M.P. 216°, corrected.

Inhibition of pyruvate formation by sodium fluoride

Exps. 47 and 54 above show that barley saps convert hexosediphosphate into pyruvic acid. Phosphoglycerate and lactate are also converted into pyruvate and either might, in theory, be an intermediate in the conversion of hexosediphosphate into pyruvate. The reaction phosphoglycerate → pyruvate, or, more exactly, its partial reaction 2-phosphoglycerate ⇆ phosphopyruvate is markedly sensitive to poisoning by sodium fluoride. Inhibition of the hexosediphosphate → pyruvate reaction by sodium fluoride would therefore suggest conversion via phosphoglycerate, whereas tolerance of this poison would rule it out.

Exp. 70. A fresh preparation of sap was divided into 3 aliquots of 40 ml. each and the usual amounts of sulphonic acid and thymol were added.

A was made up to 50 ml. with glass-distilled water;

B with a solution of hexosediphosphate (≡40 mg. Ba hexosediphosphate);

C with equivalent hexosediphosphate and NaF to a final concentration of *M*/40.

The three digests were incubated side by side for 48 hr. at 30° and alkali-soluble hydrazones isolated in the usual way. The results may be summarized as follows:

Table 1

	Digest	Distillation	Products
A	Sap + water	Complete below 50°	Partly crystalline, reddish. Narrow zone spindle-shaped crystals
B	Sap + hexosediphosphate	Temp. rose above 50°	Much larger and yellower deposit than A
C	Sap + hexosediphosphate + *M*/40 NaF	Complete below 50°	Quantity and colour very similar to A

The experiment suggested a marked inhibition due to sodium fluoride, but was necessarily inconclusive because the amount of product could only be determined by inspection, and because it was found that amounts of pyruvic acid added to sap could not be quantitatively recovered by the distillation. We therefore sought for means of estimating pyruvic acid formation more exactly. Lu's [1939] method, utilizing the colour of the hydrazone in strongly alkaline solution, was found to be useless because of the presence of varying amounts of other substances in the sap which gave intense colours and which could not be separated without serious loss of pyruvate. Straub's [1936] *o*-hydroxybenzylidene-pyruvate method suffered from the same disadvantage when applied to barley sap, but occasionally a sample of sap was obtained in which the interfering substances were present in relatively small amounts. Graded additions of pyruvic acid could then be added and a calibration graph constructed for that particular sap. This proved extremely laborious, since many samples of sap had to be examined before a suitable one was found. The first two suitable saps obtained gave results which confirmed the suggestion of the qualitative experiment above.

Exps. 56 and 63. The sap was prepared in the usual way, and the digests were set up as described below (see Table 2): Digest A was fixed and estimated immediately. Digests B and C were incubated for 46 hr. in the presence of thymol; they were then cleared with barium and trichloroacetic acid by the same method as was used in the isolation experiments, and each digest was made up to 10 ml. Aliquots of 4 ml. were taken from these and 4 ml. of salicylic aldehyde in alcohol and 2 ml. of 166 % KOH were added. The red colour of *o*-hydroxybenzylidene pyruvate was then developed by warming at 37° for 10 min., cooling and centrifuging exactly as described by Straub. The colorimetry was carried out with a Spekker absorptiometer, using a No. 6 (blue) screen. The results are given as arbitrary readings from the instrument, since the colour of the controls is not due to pyruvic acid. The assumption that the colour produced during incubation is in the main due to pyruvic acid is based on the results of the isolation experiments. The instrument numbers rise with increasing concentration of the coloured compound, though not necessarily in strict proportion.

Table 2

	Sap ml.	Hexosediphosphate ml.	1-Naphthol-2-sulphonic acid ml.	NaF ml.	Water ml.	Readings Exp. 56	Readings Exp. 63
A	2	1	—	—	1·0	322	298
			0·5	—	0·5	—	—
B	2	1	0·5	—	0·5	444	429
C	2	1	0·5	0·5	—	297	399

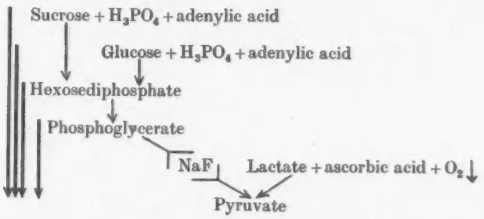
Final concentrations: NaF = $M/40$, 1-naphthol-2-sulphonic acid = 0·1 %. In Exp. 56 the hexosediphosphate added was equivalent to 3 mg. pyruvic acid and, making the necessary assumptions, the pyruvic acid estimated after incubation of digest B was about 1·2 mg.

In both the experiments the presence of NaF (digest C) has led to a smaller production of 'pyruvic acid' than occurred in its absence (digest B). In Exp. 63, inhibition was only partial at the concentration used, but in Exp. 56, at the same concentration, it was complete.

Discussion

It is a commonly held belief that the mechanism of glycolysis in the higher plants is similar to that of alcoholic fermentation in yeast. The experiments described above show that barley saps are able to promote many of the reactions now believed to occur in fermentation. They do not, strictly speaking, show that these reactions occur normally in the living barley plant, but the corresponding evidence is rather scanty, even for yeast. We have, however, shown by other methods (papers quoted in the introduction) that some at least of these reactions are likely to be taking place in living barley tissues. Our experiments were all carried out aerobically and a final formation of alcohol was, therefore, not to be expected. The exact effect of oxygen is reserved for future consideration, but it is a matter of interest that these glycolytic reactions would take place in its presence.

The reactions we have actually shown to be catalysed by barley sap are indicated by the heavy side arrows in the following diagram. The formation of pyruvate from lactate differs from the other reactions in consuming oxygen [James & Cragg, 1940], whereas they only tolerate it.



The following remarks apply to the glycolytic reactions in barley systems.

Sucrose → pyruvate. In saps with low phosphate concentrations, this reaction fails to occur [Exp. 50], but it may be brought about in saps with a normal content of inorganic phosphate by the addition of adenylic acid [Exp. 68A]. Adenylic acid has not yet been isolated from barley, but the presence of a labile phosphate carrier is suggested by the fact that the organic esters contain a small readily hydrolysable fraction [Arney, 1939]. This small amount might be rendered inoperative in the extracted saps by dilution.

Glucose → pyruvate. This reaction presents the same general characteristics as the sucrose → pyruvate reaction, i.e. it will only occur if means are provided

for phosphorylation [Exp. 69]. It is therefore a reasonable assumption that both sugars are converted into phosphate esters before their actual splitting. Barley saps always contain a relatively large amount of free phosphate unless special steps are taken for its removal. We do not at present know whether this is continuously drawn upon for phosphorylation, or whether a closed cycle, excluding free phosphate, is established.

Hexosediphosphate → pyruvate. It is probable that a plexus of sugar-phosphate esters occurs in barley as in other plants and organisms. Hexosediphosphate is likely to be one of the esters and probably the most abundant. This is indicated by analysis of the phosphate fractions by the usual methods [James & Heard, 1940]. Added hexosediphosphate is decomposed [James & Heard, 1940] with formation of pyruvate by barley saps (Exps. 54, 56, 63, 70) without addition of adenylic acid or any other agent, even when free inorganic phosphate has been precipitated beforehand (Exp. 47). Inhibition of this reaction by *M*/40 NaF (Exps. 56, 63, 70) suggests that its course lies by way of phosphoglyeerate and phosphopyruvate. Another probable stage, that involving triosephosphate, is not considered here, since the evidence collected depends on other methods.

Phosphoglycerate → pyruvate. Phosphoglycerate has not yet been isolated from barley. Saps breaking down hexosediphosphate in the presence of NaF accumulate a phosphate ester highly resistant to acid hydrolysis which is likely to be phosphoglycerate [James & Heard, 1940]. Added phosphoglycerate in the absence of NaF gave pyruvate (Exp. 67).

Summary

1. Cell-free barley saps were prepared by a simple method. On incubation for 48 hr. at 30° in the presence of thymol and 1-naphthol-2-sulphonic acid, they gave rise to little or no pyruvic acid.

2. Addition of sucrose did not cause pyruvic acid formation. Glucose and fructose would be present in the sap itself, and therefore were also incapable of giving pyruvic acid.

3. Addition of adenylic acid to starved sap (i.e. sap low in sugar-content) gave traces of what was probably pyruvic acid.

4. Addition of sucrose + adenylic acid, or glucose + adenylic acid, led to the formation of readily identifiable quantities of pyruvic acid.

5. Hexosediphosphate and phosphoglycerate were also converted into pyruvic acid. Pyruvate formation from hexosediphosphate was markedly inhibited by *M*/40 NaF.

6. Lactic acid was oxidized to pyruvic acid in the presence of ascorbic acid and atmospheric oxygen.

These results, 1–5, indicate a conversion of sugars into pyruvic acid through a phosphorylating cycle similar, at least in outline, to the reactions of yeast.

We wish to acknowledge the assistance of Dr Margaret Cattle in the latter part of the work.

REFERENCES

Arney (1939). *Biochem J.* **33**, 1078.
James & Arney (1939). *New Phytol.* **38**, 340.
—— & Cragg (1940). *New Phytol.* **39**, 334.
—— & Heard (1940). *New Phytol.* **39**, 334.
—— & James (1940). *New Phytol.* **39**, 266.
—— & Norval (1938). *New Phytol.* **37**, 455.
Lu (1939). *Biochem. J.* **33**, 249.
Peters & Thompson (1934). *Biochem. J.* **28**, 916.
Straub (1936). *Hoppe-Seyl. Z.* **244**, 117.